Gene Knockdown of γ-Glutamylcysteine Synthetase by RNAi 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, RNAi methods were used to knock down gene expression of γ-GCS in procyclic T. brucei cells. Induction of γ-GCS RNAi, with tetracycline led to cell death within 4–6 days post-induction. Cell death was preceded by the depletion of the γ-GCS protein from RNAi 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 RNAi 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 RNAi; 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 RNAi cell lines.

African trypanosomiasis is caused by subspecies of the genus, trypanosomatidae. These unflagellated 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); N3,N6-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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were grown in the presence of varying concentrations of BSO (0–150 

µM), or without the addition of BSO, and at 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-diethylthiazol-2-yl)-5-(3-carboxy-methyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron acceptant (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 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 RNAi plasmid. The pZJM vector was digested with XhoI 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 RNAi cells, 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 TbI2.1 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 with PCR with the following primers: 5′-CCACGTTTCACCGGGGCTT-CCTGATGCCG-3′ (has NotI site) and 5′-CCCTGATACAACACCGCTAGATGGTGGCCG-3′ (has MstI site). The 3′ γ-GCS fragment (nucleotide residues 6411–6797) was generated by PCR with the following primers: 5′-CCAGTTGTCGACCGCTCCATT-CATGAG-3′ (has NotI site) and 5′-CCATGTATCGACGATGCTGCGTTTTCC-3′ (has SphI site). The vector and PCR products were digested with the restriction enzymes mentioned above. The PCR products were ligated into the ends of the pHDI 228 vector. The single knockout plasmid was transfected into wild-type 427 procyclic 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 (~105–107 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 × 106 cells/ml. Aliquots of 1 x 106 cells/ml in 0.5 ml of ZPFM with 20 µg of linear DNA (vector with NotI) 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 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 (10 µg/ml) to the culture media.

**Cell Lysate Preparation and Immunoblotting**—Cells (100 ml) of procyclic trypanosomes for about 4 days were grown to 107 cells/ml (10–107 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 NaHPO4, 1.8 mM KH2PO4). Pellets were resus-
**RESULTS**

**Induction of γ-GCS RNA, 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; methods using the tetracycline-inducible pZJM dual T7 vector (30). Procyclic 29-13 cells stably transformed with the γ-GCS RNAi construct (Fig. 2A) arrested cell division after a limited number of cell cycles when induced with Tet (1 μM). After about 4 days in culture, the γ-GCS RNAi Tet cells (Fig. 2B, open circles) began to die, while the uninduced RNAi, (− 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 RNAi cell lines. Northern blot (Fig. 2B, inset, N panel) and Western blot analyses (Fig. 2B, inset, W panel) revealed that both RNA and protein levels in the induced γ-GCS RNAi cells, (+ Tet) were reduced >80%. The levels of tubulin RNA (Fig. 2B, 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 RNAi (Fig. 3A, black bars).

In addition to the γ-GCS RNAi 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. 3A, black bars).

Thiol levels in cell lysates of RNAi 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 (mmol/10^8...
cells) were determined for glutathione (GSH, peak B), glutathionyl-spermidine (GSH-SPD, peak C), and trypanothione (TSH₂, peak D) (Table I). Uninduced (~Tet) RNAi cells had 2.15 nmol of GSH, 0.79 nmol of GSH-SPD, and 3.81 nmol of TSH₂. After 2 days of induction (+Tet) 78% GSH (1.69 nmol), 46% GSH-SPD (0.36 nmol), and 32% TSH₂ (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 RNAi 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 RNAi cell death phenotype, Tet-induced γ-GCS RNAi cells were cultured in the presence of 80 μM GSH (Fig. 2B, 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 RNAi, and 29-13 parent cells) for the first 10 days of culture, 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 RNAi (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
The amounts of thiol are given as nanomoles per 10⁸ cells. The samples were analyzed in duplicate.

<table>
<thead>
<tr>
<th>Days of induction</th>
<th>Addition of GSH</th>
<th>Thiol</th>
<th>GSH</th>
<th>GSH-SPD</th>
<th>T(SH)₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td></td>
<td>2.15</td>
<td>0.79</td>
<td>3.91</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td></td>
<td>1.69</td>
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<td>2.05</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td></td>
<td>0.37</td>
<td>ND</td>
<td>0.31</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td></td>
<td>2.29</td>
<td>0.91</td>
<td>4.20</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td></td>
<td>1.82</td>
<td>0.65</td>
<td>2.49</td>
</tr>
</tbody>
</table>

a Addition of 1 μg/ml tetracycline.

b Addition of 80 μM GSH into culture media.

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 (IC₅₀) 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 (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 IC₅₀ 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 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 two pathways (40, 45). Thus the finding that GSH is an essential gene in procyclic T. brucei parasites will likely extend to bloodstream parasites, and the RNAi analysis of the procyclic cells provides support for the hypothesis that γ-GCS is an essential gene in procyclic T. brucei (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 RNAi 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 RNAi 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 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 RNAi 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, 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
in direct experimental evidence for or to rescue the cells from BSO-induced death. Although no inability of exogenous GSH to restore intracellular thiol pools would account for the likely present in ing and transporting peptide components of GSH into the cell is sulfone into the kidney (46). A similar mechanism for degradation of T. brucei –Glu–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.

Acknowledgments—We thank Alan Fairlamb for generously sharing the protocols for the thiol measurements and for providing the mono-bromobimane-labeled TSH₃ standard. We are grateful to Christine Clayton for the plasmid pDH28 expression vector, to Paul Englund for the p2ZM vector, and to George Cross for the 29-13 cells. We also thank Traci Kinkel for help with RNA isolation and cell culturing.

REFERENCES

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.

<table>
<thead>
<tr>
<th>Concentration of BSO in media</th>
<th>Addition of GSH</th>
<th>Thiol</th>
<th>GSH</th>
<th>GSH-SPD</th>
<th>TSH₂GCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>–</td>
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<td>0.68</td>
<td>3.39</td>
<td></td>
</tr>
<tr>
<td>30</td>
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<td>1.75</td>
<td>0.46</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>–</td>
<td>0.92</td>
<td>0.30</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>–</td>
<td>0.78</td>
<td>0.23</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>–</td>
<td>0.45</td>
<td>0.19</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>2.73</td>
<td>0.58</td>
<td>3.79</td>
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<tr>
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<td>+</td>
<td>1.78</td>
<td>0.65</td>
<td>2.31</td>
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<tr>
<td>60</td>
<td>+</td>
<td>1.34</td>
<td>0.56</td>
<td>1.31</td>
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</tr>
<tr>
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<td>0.24</td>
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<tr>
<td>120</td>
<td>+</td>
<td>0.49</td>
<td>0.11</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

a Addition of 80 µM GSH into culture media.

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 TS₃H₂, 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 TS₃H₂ precursors that are synthesized, thus maintaining a balanced metabolic output between the two pathways. To examine this possibility, we depleted γ-GCS levels (by RNAi) 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 TS₃H₂ 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.

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.