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# Glutaredoxin-deficiency confers bloodstream *Trypanosoma brucei* with improved thermotolerance

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ABSTRACT

As constituents of their unusual trypanothione-based thiol metabolism, African trypanosomes express two dithiol glutaredoxins (Grxs), a cytosolic Grx1 and a mitochondrial Grx2, with so far unknown biological functions. As revealed by gel shift assays, in the mammalian bloodstream form of *Trypanosoma brucei*, Grx1 is in the fully reduced state. Upon diamide treatment of the cells, Grx1 forms an active site disulfide bridge that is rapidly re-reduced after stress removal; Cys76, a conserved non-active site Cys remains in the thiol state. Deletion of both *grx*1 alleles does not result in any proliferation defect of neither the procyclic insect form nor the bloodstream form, even not under various stress conditions. In addition, the Grx1-deficient parasites are fully infectious in the mouse model. A functional compensation by Grx2 is unlikely as identical levels of Grx2 were found in wildtype and Grx1-deficient cells. In the classical hydroxyethyl disulfide assay, Grx1-deficient bloodstream cells display 50–60% of the activity of wildtype cells indicating that the cytosolic oxidoreductase accounts for a major part of the total deglutathionylation capacity of the parasite. Intriguingly, at elevated temperature, proliferation of the Grx1-deficient bloodstream parasites is significantly less affected compared to wildtype cells. When cultured for three days at 39 °C, only 51% of the cells in the wildtype population retained normal morphology with single mitochondrial and nuclear DNA (1K1N), whereas 27% of the cells displayed ≥2K2N. In comparison, 64% of the Grx1-deficient cells kept the 1K1N phenotype and only 18% had ≥2K2N. The data suggest that Grx1 plays a role in the regulation of the thermotolerance of the parasites by (in)directly interfering with the progression of the cell cycle, a process that may comprise protein (de)glutathionylation step(s).

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## 1. Introduction

Glutaredoxins (Grxs) are ubiquitous small thiol proteins that belong to the thioredoxin-fold superfamily of proteins and play crucial roles in the redox homeostasis of the cell (for reviews see Ref. [1,2]. All organisms have an individual set of isoforms that occur in the cytosol, mitochondria or nucleus of the cell. Based on the primary structure, two main groups are distinguishable, dithiol Grxs with a CXXC active site sequence (mostly CPYC) and monothiol Grxs (1-C-Grxs) containing one or more CXXS motif(s) (mostly CGFS) [3,4]. Many of the 1-C-Grxs and some dithiol Grxs coordinate iron-sulfur clusters and are involved in the biosynthesis of iron-sulfur proteins [4,5]. Generally, the functions of Grxs are closely linked with the glutathione (GSH) system. Dithiol Grxs participate in a large number of biological processes; for instance, as thioredoxins, Grxs deliver the reducing equivalents for the synthesis of DNA precursors by ribonucleotide reductase. The oxidized form generated in the reaction is then re-reduced by the GSH/glutathione reductase system. The most specific function of dithiol Grxs is probably their ability to catalyze the reversible *S*-glutathionylation of cysteine residues. This cellular mechanism can be employed to protect distinct proteins from irreversible overoxidation and, most importantly, for redox signaling purposes, thus using GSH as a redox currency [2,6,7].

Trypanosomes and *Leishmania* lack glutathione reductases and thioredoxin reductases. The parasite thiol redox metabolism is based on the low molecular mass dithiol trypanothione [bis(glutathionylspermidine, $T(SH)_2$)] and trypanothione reductase (for reviews see Ref. [8–10]). The $T(SH)_2$ system is involved in the synthesis of DNA precursors as well as the detoxification of hydroperoxides by different peroxidases reactions, mediated by tryparedoxin. This essential and parasite-specific oxidoreductase, which is a distant member of the thioredoxin-type protein family, takes over many of the functions known to be catalyzed by

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thioredoxins and/or Grxs in other organisms [11,12]. Yet, despite the absence of a classical glutathione metabolism, trypanosomatids contain appreciable concentrations of free GSH as well as a repertoire of distinct Grxs [8,13]. *Trypanosoma brucei*, the causative agent of African sleeping sickness, encodes three genes for 1-C-Grxs as well as two genes for dithiol Grxs. The 1-C-Grxs are located in the mitochondrion and cytosol, respectively, and *in vitro*, all three proteins are able to complex an iron sulfur cluster [13,14].

The dithiol Grx1 (TriTryp DB, Tb927.11.1370; Tb427tmp.47.0012) is a cytosolic protein in both the mammalian bloodstream form and procyclic insect form of *T. brucei*. In contrast, Grx2 occurs in the intermembrane space of the parasite mitochondrion ([15], Ebersoll and Krauth-Siegel, unpublished data) and thus displays a localization reported for a sub-fraction of the cytosolic Grx1 in rat hearts [16]. *T. brucei* Grx1 has an overall sequence identity of 40% with human Grx2 but harbors the active site motif (CPYC) of human Grx1. The parasite Grx2 has an unusual CQFC active site, shares only 25% of its residues with Grx1 and presents sequence features exclusively found in the trypanosomatid lineage [13]. *Trypanosoma cruzi*, the causative agent of Chagas' disease, encodes a single *grx* gene which has an overall sequence identity of 80% with *T. brucei* Grx2 but is located in the cytosol [17]. The catalytic properties of the *T. brucei* Grxs as well as *T. cruzi* Grx have been studied in some detail [15,17,18]. The dithiol forms of the proteins are readily regenerated by $T(SH)_2$, the reactions being at least three orders of magnitude faster compared to those with GSH [15,17]. The recombinant trypanosomal Grxs catalyze the reduction of GSSG by $T(SH)_2$, which underlines their close link with the trypanothione metabolism. Both *T. brucei* Grxs and the *T. cruzi* Grx catalyze the reduction of the mixed disulfide between GSH and either 2-mercaptoethanol (hydroxyethyl disulfide, HED) or cysteine residues of various model proteins. In the case of *T. brucei* Grx1, the $k_{cat}/K_m$ values correspond to those reported for human Grx1 and Grx2 [19]. The capacity of Grxs to reduce protein-GSH mixed disulfide cannot be taken over, at least to a physiological competent level, by tryparedoxin [11,15]. Similar to Grxs from other species, the *T. brucei* Grxs catalyze the reduction of protein disulfides, such as insulin disulfide used as model substrate, albeit with much lower efficiency compared to thioredoxins [15]. Interestingly, recombinant Grx1 is able to coordinate an iron-sulfur cluster that negatively regulates its redox activity [15,20]. In an attempt to disclose the Grx function in trypanosomes, overexpression of Grx2 in the epimastigote insect stage of *T. cruzi* was shown to confer resistance towards exogenous hydrogen peroxide, yet induced apoptotic phenotypes as well [17]. Thus, Grx-mediated protein modifications may be part of cell death mechanisms induced by specific stresses. Neither the specific RNA-interference against Grx1 in *T. brucei* [15] nor the genome wide approach [21] revealed any proliferation phenotype in the mammalian bloodstream (BS) form and procyclic (PC) insect form that multiplies in the tsetse fly. The biological role of the protein remained thus elusive as well as its relevance for sustaining pathogen survival in an animal host.

Here we report on the generation of BS and PC *T. brucei* in which both *grx*1 alleles were replaced by resistance genes yielding stable Grx1 knockout (KO) cell lines. We show that Grx1 is a major contributor to the total deglutathionylation capacity of the parasite. Although under normal culture conditions, the Grx1 KO cells are fully viable and even infectious in the mouse model, they display a remarkable phenotype when the culture temperature is raised. At 39 °C, BS Grx1 KO cells retain a significantly higher proliferation rate and normal morphology compared to wildtype (WT) controls suggesting that the cytosolic oxidoreductase is involved in processes that affect cell cycle progression under elevated temperatures.

## 2. Materials and methods

### 2.1. Materials

Deferoxamine mesylate, $Fe(NO_3)_3{\cdot}9H_2O$, $FeSO_4{\cdot}7H_2O$, phleomycin, *N*-ethylmaleimide (NEM), tetracycline (tet) and HED were purchased from Sigma–Aldrich, Steinheim. Puromycin dihydrochloride and blasticidin hydrochloride were ordered from Roth, Karlsruhe. Fetal calf serum (FCS) was from Biochrome and $H_2O_2$ from Merck, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) from Serva, Heidelberg, and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) from Life Technologies, Darmstadt. Recombinant *T. brucei* Grx1 was prepared following a published procedure [15] and stored at 4 °C. Polyclonal guinea pig antisera against *T. brucei* Grx1, *T. brucei* Grx2 and the rabbit antiserum against *T. brucei* cytosolic 2-Cys-peroxiredoxin (TXNPx) were obtained previously [15]. Monoclonal anti-myc antibodies were from Roche. Goat anti guinea pig or mouse IgG were purchased from Santa Cruz Biotechnology, goat anti rabbit IgG were from Thermo Fisher and Santa Cruz Biotechnology. Goat anti rat IgG were from Jackson Immuno Research. The vectors pHD1747 and pHD1748 as well as the rabbit antiserum against *T. brucei* aldolase and rat antiserum against the S9 ribosomal protein were kindly provided by Dr. Christine Clayton (ZMBH, Heidelberg). All primers were synthesized by Eurofins MWG Operons, Ebersberg, Germany. The plasmids were sequenced by EuroFins MWG Operons, Ebersberg, or GATC Biotech AG, Konstanz, Germany.

### 2.2. Cultivation of T. brucei

The parasites used in this work were of the *T. brucei* 449 cell line which is a descendant of the Lister 427 strain [22]. BS parasites were cultivated in HMI-9 medium at 37 °C in a humidified incubator with 5% $CO_2$. PC cells were grown at 27 °C in MEM-Pros medium. Both media were supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. In addition, the HMI-9 medium and MEM-Pros medium contained 0.2 μg/ml and 0.5 μg/ml, respectively, of phleomycin to select for cells containing the tetracycline (tet) repressor gene (for more details see Refs. [14,23]).

### 2.3. Cloning of the constructs for the deletion of grx1 in T. brucei

Genomic DNA was isolated from $5 \times 10^6$ bloodstream *T. brucei* using the Qiagen DNeasy Blood and Tissue kit. The 5′ untranslated region (UTR) of *grx1* was amplified by PCR with *Pfu* polymerase and 5UTR-5aF-*grx1*-XhoI and 5UTR-5bR-*grx1*-HindIII as primers (Table 1). The 3′ UTR was amplified using the primers 3UTR-3aF-*grx1*-EcoRI and 3UTR-3bR-*grx1*-NotI. The fragments were purified from a 2% w/v agarose gel using the Zymoclean Gel DNA Recovery kit and ligated into the pGEM-T vector according to the manufacturer's protocol (pGEM-T and pGEM-T Easy Vector Systems) and amplified in NovaBlue cells. Plasmid DNA was isolated using the NucleoBond Extra Midi kit. The 5′ and 3′UTRs were released from the plasmid by digestion with XhoI/HindIII and EcoRI/NotI, respectively, and stepwise cloned into the pHD1747 (confers resistance to puromycin) and pHD1748 (blasticidin) vectors to generate pHD1747-*grx1*-KO and pHD1748-*grx1*-KO, respectively. The XhoI and NotI digested pHD1747-*grx1*-KO and pHD1748-*grx1*-KO cassettes had a size of 1375 bp and 1171 bp, respectively.

### 2.4. Generation of Grx1 KO cell lines

12 μg of pHD1747-*grx1*-KO and pHD1748-*grx1*-KO, respectively, were digested with XhoI and NotI. The digest (50 μl) was diluted to 100 μl with sterile water. 100 μl of 4 M ammonium

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