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Research paper

A glutaredoxin in the mitochondrial intermembrane space has stage-specific functions in the thermo-tolerance and proliferation of African trypanosomes

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ABSTRACT

Trypanosoma brucei glutaredoxin 2 (Grx2) is a dithiol glutaredoxin that is specifically located in the mitochondrial intermembrane space. Bloodstream form parasites lacking Grx2 or both, Grx2 and the cytosolic Grx1, are viable *in vitro* and infectious to mice suggesting that neither oxidoreductase is needed for survival or infectivity to mammals. A 37 °C to 39 °C shift changes the cellular redox milieu of bloodstream cells to more oxidizing conditions and induces a significantly stronger growth arrest in wildtype parasites compared to the mutant cells. Grx2-deficient cells ectopically expressing the wildtype form of Grx2 with its C31QFC34 active site, but not the C34S mutant, regain the sensitivity of the parental strain, indicating that the physiological role of Grx2 requires both active site cysteines. In the procyclic insect stage of the parasite, Grx2 is essential. Both alleles can be replaced if procyclic cells ectopically express authentic or C34S, but not C31S/C34S Grx2, pointing to a redox role that relies on a monothiol mechanism. RNA-interference against Grx2 causes a virtually irreversible proliferation defect. The cells adopt an elongated morphology but do not show any significant alteration in the cell cycle. The growth retardation is attenuated by high glucose concentrations. Under these conditions, procyclic cells obtain ATP by substrate level phosphorylation suggesting that Grx2 might regulate a respiratory chain component.

1. Introduction

Glutaredoxins (Grxs) are ubiquitous small oxidoreductases that belong to the thioredoxin superfamily and play crucial roles in the redox homeostasis of the cell (for reviews see [1,21,65]). All organisms have an individual set of isoforms that occur in the cytosol, mitochondria and nucleus. Based on the primary structure, two main groups are distinguishable, monothiol Grxs containing one or more CXXS motif(s) and the more classical dithiol Grxs with a CXXC active site sequence (mostly CPYC) [36]. Dithiol Grxs were first identified as highly efficient electron donors for ribonucleotide reductase and thus for the synthesis of the DNA building blocks [36,66]. Like thioredoxins, dithiol Grxs participate in a wide variety of biological processes, many of them can catalyze the reduction of protein disulfides using both active site cysteine residues albeit usually at lower efficiency than thioredoxins [37]. Grxs have been shown to play distinct roles in the sensitivity of various cells towards oxidative stressors [1,10,21,39,41]. A remarkable recent finding is that limiting amounts of the cytosolic Grxs in the mitochondrial intermembrane space (IMS) of *Saccharomyces cerevisiae* provide a kinetic barrier that prevents the reduction of target proteins by glutathione (GSH) [31].

A unique feature of dithiol Grxs is their ability to catalyze redox reactions using only the first cysteine (monothiol reactions). Generally, the functions of Grxs are closely linked to the GSH system since (i) their reduced form is regenerated by thiol/disulfide exchange of the oxidized protein with GSH, where the GSSG formed is then reduced by

Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; ASCT, acetate:succinate-CoA-transferase; BS, bloodstream; cTXNPx, cytosolic 2-Cys-peroxiredoxin; GR, glutathione reductase; Grx, glutaredoxin; Gsp, glutathionylspermidine; HED, (hydroxyethyl)disulfide; KO, knockout; IMS, intermembrane space; LipDH, lipoamide dehydrogenase; 2-ME, 2-mercaptoethanol; MM(PEG)12, methyl-PEG12-maleimide; mTXNPx, mitochondrial 2-Cys-peroxiredoxin; PC, procyclic; RNAi, RNA interference; *S. cerevisiae*, *Saccharomyces cerevisiae*; SKO, single knockout; *T. brucei*, , *Trypanosoma brucei*; *T. cruzi*, *Trypanosoma cruzi*; TCEP, tris(2-carboxyethyl)phosphine; Tpx, tryparedoxin; tet, tetracycline; $T(SH)_2$, trypanothione; TS_2, trypanothione disulfide; WT, wildtype

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glutathione reductase, and (ii) they catalyze with high efficiency and selectivity the reversible S-glutathionylation of proteins. The latter mechanism may be employed to protect reactive cysteine residues in distinct proteins from irreversible over-oxidation as well as for redox signaling pathways that could mediate critical cellular functions like proliferation and apoptosis [1,21,41,65].

Trypanosomatids, such as *Trypanosoma brucei,* the causative agent of African sleeping sickness and Nagana cattle disease, lack glutathione reductases and thioredoxin reductases and their thiol metabolism is based on the low molecular mass dithiol trypanothione [bis(glutathionyl)spermidine, $T(SH)_2$] and trypanothione reductase (for reviews see [33,34,44]). $T(SH)_2$ is synthesized from two molecules of GSH that are covalently linked by spermidine with glutathionylspermidine (Gsp) as intermediate [11,51]. The $T(SH)_2$ system is involved in the synthesis of DNA precursors as well as the detoxification of hydroperoxides. The reactions are mediated by tryparedoxin (Tpx). This essential and parasite-specific oxidoreductase is a distant member of the thioredoxin-type protein family and fulfils many of the functions known to be catalyzed by thioredoxins and/or Grxs in other organisms [13,59]. Despite the absence of a classical glutathione system, trypanosomatids contain appreciable concentrations of free GSH as well as a repertoire of distinct Grxs [12,33]. Recently we showed that as response to exogenous and endogenous oxidative stresses, the mammalian bloodstream (BS) form of *T. brucei* can undergo protein S-glutathionylation and S-trypanothionylation [64].

The *T. brucei* genome encodes genes for three monothiol Grxs as well as two dithiol Grxs (Grx1 and Grx2) [12]. Grx1 represents a canonical dithiol Grx whereas Grx2 has sequence features exclusively found in trypanosomatid organisms [12]. In *T. brucei*, Grx1 is a cytosolic protein whereas Grx2 is located in the mitochondrion and preliminary studies suggest a localization in the intermembrane space (IMS) [9]. Interestingly, *Trypanosoma cruzi*, the causative agent of Chagas' disease, encodes a single *grx* gene. The protein has an overall sequence identity of 80% with *T. brucei* Grx2 and is located in the cytosol [46].

The catalytic properties of recombinant *T. brucei* Grx1 and Grx2 as well as *T. cruzi* Grx have been studied in some detail [9,46,47]. The reduced form of the proteins with the active site cysteines (Cys31 and Cys34 in *T. brucei* Grx2) in the thiol state is regenerated from the intramolecular disulfide by spontaneous thiol/disulfide exchange with $T(SH)_2$, reactions that are at least three orders of magnitude faster compared to those with GSH [9,46]. The trypanosomal Grxs accelerate the reduction of GSSG by $T(SH)_2$ which again reflects their close link with the trypanothione metabolism. Both *T. brucei* Grxs and *T. cruzi* Grx catalyze the reduction of the mixed disulfide between GSH and either 2-mercaptoethanol or cysteine residues of various model proteins, a reaction that is not taken over, at least to a physiological competent degree, by Tpx [9,43,46]. Indeed, the cytosolic Grx1 has been shown to contribute to about 50% of the deglutathionylation capacity of infective *T. brucei* [49] and facilitates the reversion of stress-induced protein S-thiolation [64]. Nonetheless, at variance with Tpx [13], parasites lacking Grx1 are fully proliferative *in vitro* and *in vivo*, and even display a marked thermoresistance when grown at 39 °C [49]. In contrast, RNA-interference (RNAi) against Grx2 causes growth retardation of procyclic (PC) insect stage cells [9] indicating life cycle specific functions of Grxs in trypanosomes. Supporting this assumption, overexpression of Grx in the amastigote intracellular form of *T. cruzi* confers resistance against oxidative damage and promotes parasite growth while in non-infective parasites it induces apoptosis [46].

Here we investigated the molecular and biological details of the overall contribution of the Grx-dependent metabolism for parasite survival in an animal host as well as of the indispensability of Grx2 for PC trypanosomes. We show that Grx2 specifically localizes to the IMS of the mitochondrion and that its biological functions require the presence of both (in BS cells) or only the first (in PC cells) of the active site cysteine residues. Grx2 was dispensable for infective trypanosomes but, as observed for Grx1 KO cells, its absence increased the thermo-tolerance of BS cells. Thus, from a therapeutic point of view, the parasite Grxs can be ruled out as putative drug target molecules. Remarkably, the temperature rise lowered the cellular $T(SH)_2/TS_2$ ratio and increased the level of S-glutathionylated proteins, whereby these effects were Grx-independent. In contrast, Grx2 is essential in PC cells. The insect stage requires a redox-active Grx2 for viability. RNAi-mediated downregulation of the oxidoreductase resulted in cells with elongated morphology that were irreversibly arrested in proliferation.

2. Material and methods

2.1. Material

Insulin, digitonin, diamide, tetracycline (tet), DAPI, (hydroxylethyl) disulfide (HED), 2-mercaptoethanol (2-ME) and sodium (meta) arsenite were purchased from Sigma-Aldrich. Puromycin dihydrochloride, hygromycin B, and blasticidin hydrochloride were from Roth, Karlsruhe. Fetal calf serum (FCS) was from Biochrome, H_2O_2, G418 and mono (bromo)bimane (mBBr) from Merck. 5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was ordered from Serva, Heidelberg, and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) from Life Technologies. The Bond Breaker tris(2-carboxyethyl)phosphine (TCEP) solution and methyl-PEG12-maleimide [MM(PEG)12] were from Thermo Scientific. Recombinant Grx2 was produced as described [9]. Recombinant human glutathione reductase (GR) was a kind gift of Dr. Heiner Schirmer, Heidelberg University. Polyclonal guinea pig antisera against *T. brucei* Grx1 as well as rabbit antisera against *T. brucei* cytosolic 2-Cys-peroxiredoxin (cTXNPx), lipoamide dehydrogenase (LipDH) and Tpx were obtained previously [9,13,58]. Polyclonal guinea pig antibodies against the mitochondrial *T. brucei* 2-Cys-peroxiredoxin (mtTXNPx) as well as rabbit and guinea pig antibodies against Grx2 were generated by Eurogentec. Polyclonal rabbit antibodies against acetate: succinate-CoA-transferase (ASCT) were a kind gift from Dr. Frédéric Bringaud, University of Bordeaux. Goat anti guinea pig IgG were purchased from Santa Cruz Biotechnology. Goat anti rabbit IgG were from Thermo Scientific or Santa Cruz Biotechnology. The pHD vectors used for *T. brucei* transfection were kindly provided by Dr. Christine Clayton, Heidelberg University. All primers were synthesized by Eurofins MWG Operons, Ebersberg, Germany. The plasmids were sequenced by Euro-Fins MWG Operons or GATC Biotech AG, Konstanz, Germany.

2.2. Cultivation of T. brucei under various conditions

The parasites used in this work were of the *T. brucei brucei* 449 cell line which is a descendant of the Lister 427 strain [17]. BS parasites were cultivated in HMI-9 medium at 37 °C in a humidified incubator with 5% CO_2. If not stated otherwise, PC cells were grown at 27 °C in MEM-Pros medium. Both media were supplemented with 10% (v/v) heat-inactivated FCS, 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 tet repressor gene [62,6]. To study the temperature sensitivity of BS parasites, WT parasites and the Grx mutants were grown at 39 °C in the presence or absence of 1 μg/ml tet in medium without hygromycin. The response of BS cells towards hydrogen peroxide, diamide and sodium arsenite was studied in HMI-9 medium lacking cysteine and 2-ME. The response of PC cells towards hydrogen peroxide and diamide was followed in SDM-79 medium. A putative temperature phenotype of Grx2-depleted PC cells was studied at 37 °C. The proliferation of PC under high glucose conditions was followed in MEM-Pros medium supplemented with 10 mM glucose. Cells were counted in a Neubauer chamber.