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Depletion of the thioredoxin homologue tryparedoxin impairs antioxidative defence in African trypanosomes

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In trypanosomes, the thioredoxin-type protein TXN (tryparedoxin) is a multi-purpose oxidoreductase that is involved in the detoxification of hydroperoxides, the synthesis of DNA precursors and the replication of the kinetoplastid DNA. African trypanosomes possess two isoforms that are localized in the cytosol and in the mitochondrion of the parasites respectively. Here we report on the biological significance of the cTXN (cytosolic TXN) of *Trypanosoma brucei* for hydroperoxide detoxification. Depending on the growth phase, the concentration of the protein is 3–7-fold higher in the parasite form infecting mammals (50–100 μM) than in the form hosted by the tsetse fly (7–34 μM). Depletion of the mRNA in bloodstream trypanosomes by RNA interference revealed the indispensability of the protein. Proliferation and viability of cultured trypanosomes were impaired when TXN was lowered to 1 μM for more than 48 h. Although the levels of glutathione, glutathionylspermidine and trypanothione were increased 2–3.5-fold, the sensitivity against exogenously generated H_2O_2 was significantly enhanced. The results prove the essential role of the cTXN and its pivotal function in the parasite defence against oxidative stress.

Key words: African trypanosome, hydroperoxide metabolism, thioredoxin, *Trypanosoma brucei*, trypanothione, tryparedoxin.

INTRODUCTION

Protozoan parasites of the genera *Trypanosoma* and *Leishmania* are classified together in a group known as haemoflagellates. Members of this group are responsible for widespread diseases that kill and disable humans and animals mainly in countries with tropical and subtropical climates. Treatment of these diseases relies on chemotherapy that can hardly be rated as satisfactory [1,2].

In general, the search for new drug targets focuses on pathways and/or components that are either not present or substantially different from their congeners in the mammalian hosts. In this context, the peroxide metabolism of trypanosomatids is an appealing source of candidates, since it depends exclusively on $T(SH)_2$ [trypanothione; N^1,N^8-bis(glutathionyl)spermidine] [3], a low-molecular-mass dithiol absent from mammals. In the parasites, many vital functions are either directly fulfilled by $T(SH)_2$ or catalysed by $T(SH)_2$-dependent enzymes (Figure 1). An important mediator for the $T(SH)_2$-fuelled redox reactions is the dithiol protein TXN (tryparedoxin). Comparative sequence analysis classifies TXN as a remote relative of the thioredoxin family of proteins, since the common motif CXXC at the active site and some other features are conserved throughout this type of oxidoreductases [9–11]. The genome of *Trypanosoma brucei* discloses three putative TXN sequences on chromosome 3: two copies of *ctxn* (accession numbers XM_819232 and XM_819230) and a single copy for a mitochondrial TXN (accession number XM_821489) [12]. *T. brucei* cTXN (cytosolic TXN) has been shown to be restricted to the cytosol [13]. The mitochondrial TXN does not contain any obvious targeting signal, but a putative transmembrane domain in its C-terminus moiety that may anchor the protein to the membrane of the mitochondrion, as it has been shown in *Leishmania infantum* [11] and inferred from expression studies in *T. brucei* [14]. Functionally, TXN acts as a multi-purpose oxidoreductase transferring the reducing equivalents from $T(SH)_2$ to a variety of protein targets (Figure 1). For instance, reduced TXN delivers electrons to: (i) peroxiredoxin- [TXNPx (2-Cys-peroxiredoxin-type TXN peroxidase)] and glutathione peroxidase-type peroxidases, which detoxify hydroperoxides and peroxynitrite [7–10,15]; (ii) ribonucleotide reductase, a key enzyme that provides deoxyribonucleotides for the synthesis and repair of DNA [16]; (iii) the UMSBP (universal minicircle sequence-binding protein) involved in the initial step of replication of the kinetoplast, the trypanosomatid mitochondrial DNA [14,17]. An RNAi (RNA interference) approach to identify pathways that are essential for oxidative defence in *T. brucei* found a phenotype in terms of impaired growth and viability for the knockdown of cTXN, cytosolic TXNPx and a glutathione peroxidase-type protein, while the mitochondrial TXN and TXNPx appeared to be less important. However, only cells depleted of the cytosolic TXNPx mRNA displayed a 16-fold higher susceptibility to exogenous H_2O_2 [18]. This result conflicted with the generally accepted view that cTXN is indispensable for cytosolic peroxide metabolism in trypanosomes (Figure 1) [9]. The existence of alternative sources of reducing equivalents or other routes of hydroperoxide detoxification was discussed to account for the failure of cTXN knockdown to affect oxidative stress response [18]. Alternatively, the cellular concentration of cTXN, which reaches 3–5 % of the total soluble protein in *Crithidia fasciculata* [9], or the inability of the RNAi system used to deplete cTXN to a critical level might have obscured the pivotal role of the protein.

The aim of the present study was to address these discrepancies by using a more reliable host/vector system, analysing gene silencing with respect to time-resolved cTXN concentrations in a larger number of clones and investigating the consequences

Abbreviations used: dsRNA, double-stranded RNA; GOD, glucose oxidase; Gsp, glutathionylspermidine; LC_{50}, concentration of compound that is lethal to 50 % of the parasites under the test conditions; RNAi, RNA interference; $T(SH)_2$, trypanothione; TXN, tryparedoxin; cTXN, cytosolic TXN; TXNPx, 2-Cys-peroxiredoxin-type TXN peroxidase; UMSBP, universal minicircle sequence-binding protein.

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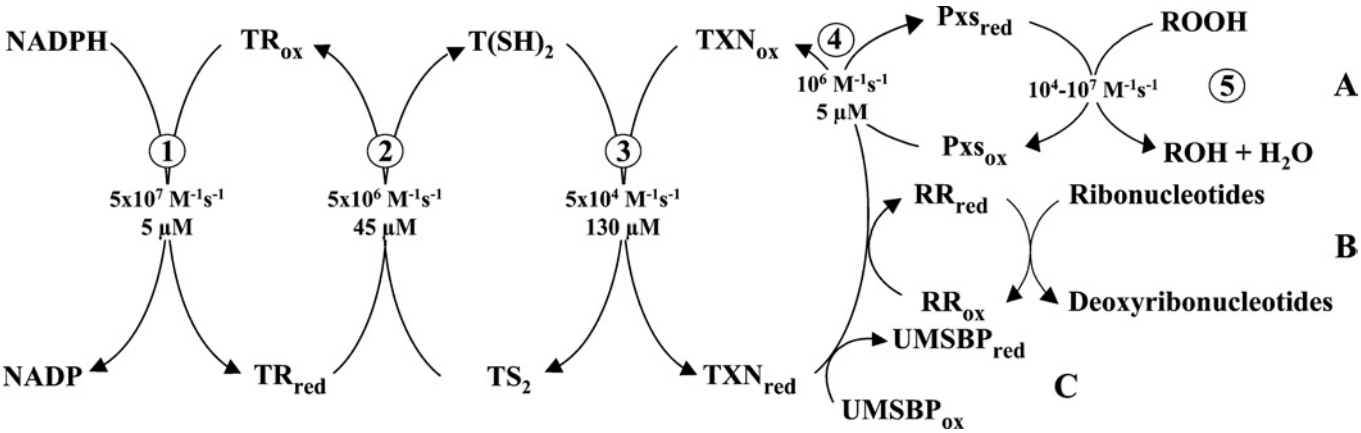

Figure 1 The TXN-mediated thiol metabolism in trypanosomatids

The redox metabolism of trypanosomatids is based onT(SH)$_2$, which is kept reduced by the NADPH-dependent flavoenzyme trypanothione reductase (TR). The dithiol T(SH)$_2$ reduces TXN that transfers the electrons to distinct target proteins. Metabolic and cellular functions that involve TXN are: (**A**) the detoxification of hydroperoxides and peroxynitrites by different types of peroxidases (peroxiredoxin- and glutathione peroxidase-type peroxidases; Pxs), (**B**) the biosynthesis of deoxyribonucleotides by ribonucleotide reductase (RR), and (**C**) the initiation of the replication process of the kinetoplast DNA by the UMSBP. Representative apparent rate constants ($M^{-1} \cdot s^{-1}$) and K_m values for individual reactions were obtained for *T. cruzi* TR [4] (reactions 1 and 2), *C. fasciculata* cTXN-1 [5] (reaction 3), *T. brucei* cTXN [6] (reaction 4), and *T. brucei* cytosolic TXNPx and *T. brucei* glutathione peroxidase-type TXN peroxidase (reaction 5) ([6,7] and [8] respectively).

of cTXN depletion on the levels of low-molecular-mass thiols. The results unequivocally verify the pivotal role of cTXN in the hydroperoxide metabolism of bloodstream forms of *T. brucei brucei*.

EXPERIMENTAL

Trypanosome growth

T. brucei brucei bloodstream and procyclic parasites were grown in HMI-9 medium [19] at 37 °C in a humidified atmosphere under 5 % CO_2 and in MEM-Pros medium (Biochrom) with 7.5 $\mu g \cdot ml^{-1}$ haemin at 27 °C respectively. Both culture media were supplemented with 10 % (v/v) fetal calf serum, 50 units $\cdot ml^{-1}$ penicillin, 50 $\mu g \cdot ml^{-1}$ streptomycin and, depending on the cell line, different selection markers (see below).

Estimation of the TXN concentration in *T. brucei*

Bloodstream or procyclic trypanosomes were harvested by centrifugation at 2000 ***g*** for 10 min at 4 °C and washed once with PBS (pH 7.0). The cells were disrupted by a single freezing–thawing step, followed by boiling in the presence of 1× SDS-loading buffer with 1.25 % (v/v) 2-mercaptoethanol. Tag-free recombinant *T. brucei* cTXN was prepared as described by Lüdemann et al. [10] and used as standard (2.5, 5, 10, 25 and 50 ng). The proteins were separated by SDS/PAGE (15 % gel) and blotted on to a Hybond-P membrane (Amersham Biosciences). The membrane was incubated with the polyclonal rabbit serum against *T. brucei* cTXN (dilution 1:2000). Anti-rabbit IgG conjugated with horseradish peroxidase (dilution 1:20 000; Santa Cruz Biotechnology) served as the secondary antibody. The immunoreaction was detected using the SuperSignal West Pico chemiluminescent substrate (Pierce). For Western-blot analysis of the RNAi clones, the membrane was simultaneously incubated with rabbit antibodies against *T. cruzi* trypanothione reductase (1:2000) as loading control. The density of the protein bands was quantified using the program BIO-PROFIL (Vilber Lourmat). A cell volume of 58 and 100 fl (femtolitres) for bloodstream [20] and procyclic trypanosomes respectively was assumed to calculate the cellular concentrations of cTXN.

RNAi constructs and transfection

The cTXN-RNAi constructs were prepared from the plasmids p2T7.ISG75a [21] and p2T7TA-177 [22] both containing two head-to-head T7 promoters, each regulated by a tetracycline operator, and a hygromycin-resistance cassette. The vectors integrate at different loci of the *T. brucei* genome. p2T7.ISG75a and p2T7TA-177 target the rRNA intergenic region and the 177 bp repeat sequence of the minichromosomes respectively. The p2T7-*ctxn* and p2T7TA-177-*ctxn* constructs were obtained by cloning the PCR fragment between bp 76 and 404 of the cTXN gene into the HindIII and BamHI, and XhoI and BamHI, restriction sites of plasmid p2T7.ISG75a and p2T7TA-177, respectively. *Escherichia coli* DH5-α cells were used for cloning and amplification of plasmid DNA. Restriction analysis and DNA sequencing confirmed the identity and integrity of the constructs. For RNAi, bloodstream *T. brucei* that encode T7 RNA polymerase (Phleomycin®) and either one or two copies of the tetracycline-repressor protein (Neomycin®), cell line 90-13 [23] or 514-1313 [24] respectively, served as host cell. Electroporation and selection of transfectants were carried out as described previously [25].

Phenotype analysis of RNAi cell lines

Synthesis of cTXN dsRNA (double-stranded RNA) was induced by adding 1 $\mu g \cdot ml^{-1}$ tetracycline to the medium. Routinely, cTXN-RNAi cell lines were seeded at 1×10^5 cells $\cdot ml^{-1}$ in the presence or absence (non-induced controls) of tetracycline. Cell growth was monitored daily and the cultures were diluted back to 1×10^5 cells $\cdot ml^{-1}$. Samples from induced and non-induced cultures were withdrawn and used for Western blotting and thiol analysis.

Thiol determination

Low-molecular-mass thiols were extracted from cells with trichloroacetic acid, derivatized with monobromobimane and

analysed by HPLC as previously described [26]. Gsp (glutathionylspermidine) disulfide and trypanothione disulfide (Bachem) were enzymatically reduced. GSH (Sigma), Gsp and $T(SH)_2$ were derivatized with monobromobimane and served as standards. Concentrations of thiols in cell extracts were calculated, considering a cell volume of 58 fl for bloodstream trypanosomes [20].

Hydroperoxide-sensitivity assays

Bloodstream trypanosomes grown for 24 h in the presence and absence of tetracycline were harvested by centrifugation at 2000 ***g*** for 10 min at 20 °C. The cell density was adjusted to 5×10^5 cells · ml^{-1} by resuspending the cell pellet in 10 ml of fresh and pre-warmed HMI-9 medium with or without tetracycline. Continuous production of H_2O_2 was achieved by adding 2, 1 and 0.5 m-units · ml^{-1} GOD (glucose oxidase; Roche). The initial rate of H_2O_2 production was determined as described by Comini et al. [25]. Under experimental conditions, 2, 1 and 0.5 m-units · ml^{-1} GOD generate H_2O_2-fluxes of 250, 124 and 62 pmol · ml^{-1} · min^{-1} respectively. For hydroperoxide bolus challenge, 1, 5, 25, 125 or 625 μM H_2O_2 was directly added to the culture. The cell cultures were incubated at 37 °C and 5 % CO_2, and cell viability was assessed after 6 and 24 h by counting living parasites in a Neubauer chamber under the light microscope. Dead parasites were distinguished by morphological and motility criteria.

Data analysis

Statistical analyses were performed by the Student's *t* test.

RESULTS

Expression of cTXN in different life stages of *T. brucei*

The expression of cTXN in procyclic and bloodstream forms of *T. brucei* was investigated by Western-blot analysis (Figure 2). Procyclic trypanosomes showed a continuous increase in the expression of cTXN over the entire time course of the culture (from 7 to 34 μM). In bloodstream parasites, the content of cTXN was doubled from the early to the mid-exponential phase (from 50 to 100 μM) and remained constant afterwards (80–90 μM). Overall, bloodstream parasites had a substantially higher concentration of cTXN (3–7-fold) than the form hosted by invertebrates irrespective of the growth phase analysed.

Growth phenotype of cTXN-RNAi cell lines

Depletion of cTXN was studied in bloodstream-form parasites, the life stage of *T. brucei* that infects mammals. Preliminary RNAi experiments were performed using the host/vector combination BSF 90-13/p2T7-*ctxn* (see Supplementary Figure 1S at http://www.BiochemJ.org/bj/402/bj4020043add.htm). Induction of cTXN-RNAi revealed that only one of six hygromycin-resistant cell lines displayed a slight growth retardation phenotype, while the remaining ones behaved similar to the non-induced controls (Supplementary Figure 1SA). Western-blot analysis of the tetracycline-responsive clone (Supplementary Figure 1SB) showed that cTXN expression was transiently (for 24–48 h) and partially (to 24–45 % of the non-induced trypanosomes) down-regulated. It is worth noting that such a failure to obtain stably regulated transformants from this system has already been reported [21,22,25].

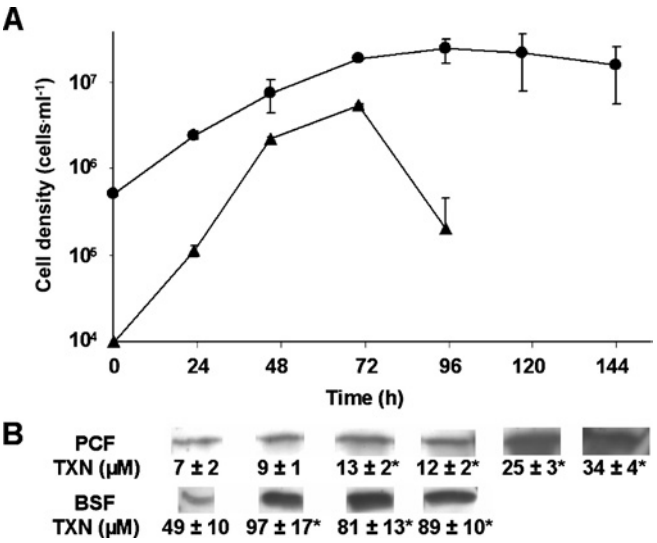

Figure 2 Expression of cTXN during different growth stages of bloodstream and procyclic *T. brucei*

(**A**) Growth curve of wild-type bloodstream (BSF; ▲) and procyclic (PCF; ●) *T. brucei* cell line 449. (**B**) At the indicated times, extracts from 5×10^5 cells were analysed by Western blotting. The concentration of cTXN was estimated by densitometric analysis using different amounts of recombinant cTXN as standard. The cTXN concentration at the different growth phases is compared with the concentration at 24 h, and significant changes ($P < 0.01$, $n = 3$, two-tailed Student's *t* test) are marked by an asterisk. Protein bands corresponding to the same gel are displayed separately.

Table 1 Concentration of cTXN in RNAi-induced bloodstream *T. brucei*

The results are the means ± S.D. for each clone tested (B1, B6, C1, C6 and D5).

Time (h) post RNAi induction	TXN (μM)
0	37.0 ± 19.1
24	1.0 ± 0.4
48	1.1 ± 1.2
240	69.6 ± 19.4

Transfection of BSF 514-1313 with the vector p2T7TA-177-*ctxn* and subsequent selection with hygromycin yielded seven resistant clones. In five cell lines, expression of cTXN was consistently down-regulated after addition of tetracycline (Figure 3), while the remaining two clones were refractory (results not shown). Induction of cTXN-RNAi did not affect cell growth within the first 24 h, but, if sustained for the next 24 h, significantly lowered cell proliferation. The doubling time was 16 ± 2 and 8 ± 1 h for the tetracycline-induced and non-induced cultures respectively (Figure 3A). After 48 h of continuous cTXN-down-regulation, cell viability was markedly impaired, giving rise to significant cell death in the cultures, a phenomenon that lasted for the next 48 h. Western-blot analysis showed substantial clonal variation in the cTXN concentrations in the RNAi-non-induced state: 58, 53, 42, 20 and 16 μM for the clones B1, B6, C6, C1 and D5 respectively (mean 37 ± 19 μM; Table 1). These differences may be due to a discrete leakage of the RNAi-system generating dsRNA in the absence of inducer [22]. After 24 h of growth in the presence of tetracycline, the cTXN concentration was reduced to approx. 1 μM (Table 1), which shows that depletion of cTXN precedes growth inhibition by 24 h (compare Table 1 and Figure 3A). Despite the severe growth retardation between 48 and 96 h, complete cell death was never observed, and after 96 h of continuous cultivation in the presence of tetracycline,

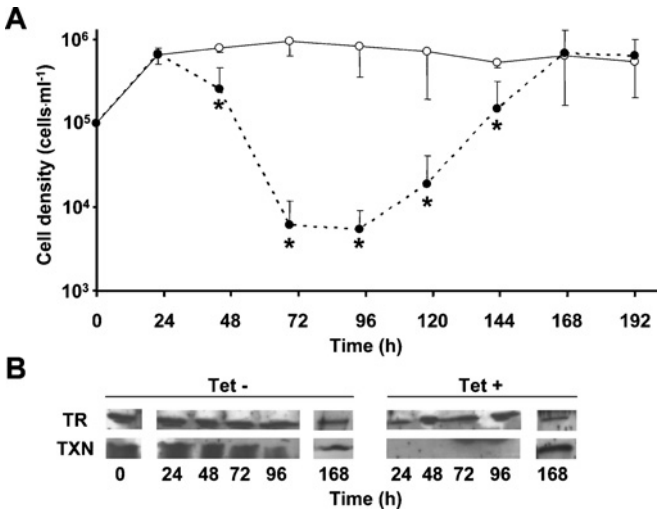

Figure 3 Growth phenotype of cTXN-depleted bloodstream trypanosomes

(**A**) The growth of cTXN-RNAi cell lines (clones B1, B6, C1, C6 and D5) was monitored in the presence (●) and absence (○) of tetracycline. The cell densities represent the average of all clones evaluated with the corresponding standard deviations. During the first 24 h after RNAi induction, no obvious growth phenotype was observed. Within the following 5 days (48–144 h), the continuous induction of RNAi significantly affected cell proliferation and viability ($P < 0.05$, $n = 5$, two-tailed Student's t test). After 168 h, the RNAi-induced cell lines reverted the growth phenotype. Asterisks (*) mark values that significantly differ between tetracycline-induced and non-induced cell lines. (**B**) Representative Western blot (clone B6) of RNAi-induced (Tet+) and non-induced cells (Tet–). 5×10^5 cells were applied per lane. cTXN could not be detected within the first 4 days after induction. Reversion of growth phenotype was associated with recovery of cTXN expression (168 h, Tet+). The simultaneous immunostaining of trypanothione reductase (TR) served as loading control. Protein bands corresponding to the same gel are displayed separately. Tet, tetracycline.

revertant trypanosomes appeared (Figure 3A). cTXN levels in these parasites either reached or even surpassed those of the non-induced cells (Figure 3B). Clones B1, B6 and C6, for instance, showed a cTXN concentration of 66 ± 7 μM, which corresponds to that of the non-induced state (50 ± 11 μM), while clones D5 and C1 had a 3–5-fold higher concentration of cTXN (47 ± 3 and 100 ± 7 μM respectively) compared with the parasites grown in the absence of tetracycline (18 μM). Microscopical examination of the tetracycline-responsive clones revealed that cells cultivated under optimal conditions but depleted of cTXN consistently had a reduced size and impaired motility. The onset of such phenotypical alterations appeared to be related to the basal concentration of cTXN, since abnormal morphologies were detected after 48 h of RNAi in cell lines D5 and C1, but only after 72 h in the B1, B6 and C6 clones.

Because the BSF 514-1313/p2T7TA-177-*ctxn* host/vector couple yielded a large number of clones displaying an efficient RNAi of cTXN, cell lines originated from this system were selected for further studies.

Thiol content in cTXN-knockdown cells

The concentration of free thiols in the parasites was determined by HPLC analysis after derivatization with monobromobimane. At the early exponential phase, the concentration of GSH, Gsp and $T(SH)_2$ in non-induced cell lines was 305 ± 65, 12 ± 7 and 149 ± 60 μM respectively (Figure 4). Down-regulation of cTXN-expression for 24 h was accompanied by an increase in the three thiols that reached statistical significance in the case of Gsp and $T(SH)_2$ (Figures 4B and 4C). Increase in GSH levels became significant 48 h after tetracycline induction (Figure 4A).

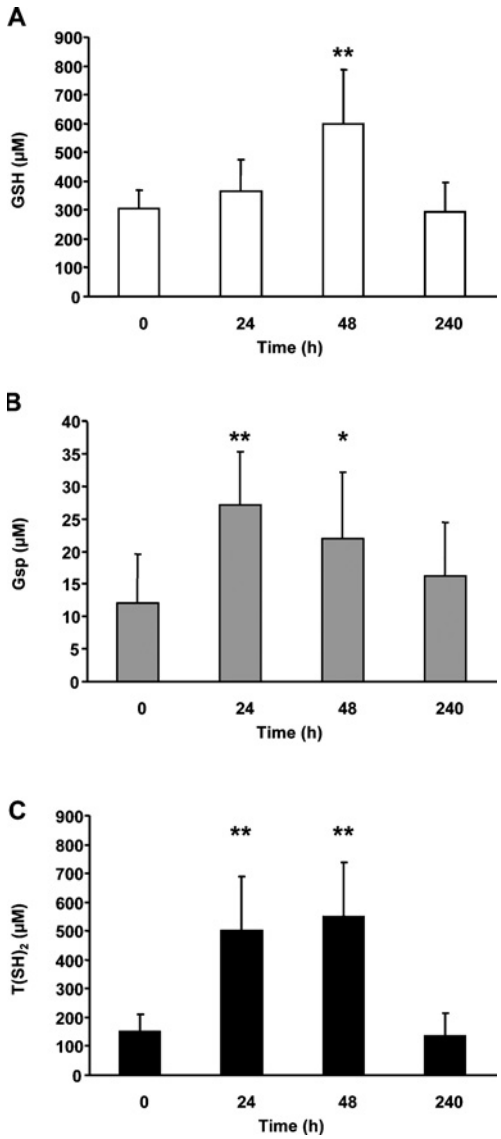

Figure 4 Thiol concentration in cTXN-RNAi bloodstream trypanosomes

Glutathione (**A**), Gsp (**B**) and $T(SH)_2$ (**C**) from cell extracts of cTXN-RNAi cell lines cultured in the presence or absence of tetracycline were separated and quantified by HPLC analysis as described in the Experimental section. The results shown are the means for the averaged duplicate values of the three clones tested (B1, B6 and C1) with standard deviations. Asterisks denote significant differences with respect to non-induced controls with $P < 0.05$ (*) or $P < 0.01$ (**) ($n = 3$; two-tailed Student's t test).

At this time point, Gsp and $T(SH)_2$ levels remained significantly elevated when compared with the non-induced cells, but did not significantly differ from the 24 h values. The thiol concentrations in the revertant parasites were comparable with those of the non-induced state. In summary, 24–48 h after RNAi induction, the concentration of GSH and Gsp was approx. 2 and that of $T(SH)_2$ 4 times higher than that in control trypanosomes. This indicates that depletion of cTXN leads to an increased synthesis of the low-molecular-mass thiols, which transiently can compensate for the lack of the dithiol protein.

Sensitivity of cTXN-RNAi cell lines towards oxidative stress

Trypanosomes depleted of cTXN were subjected to oxidative stress produced by either a steady flux or a bolus of H_2O_2. In

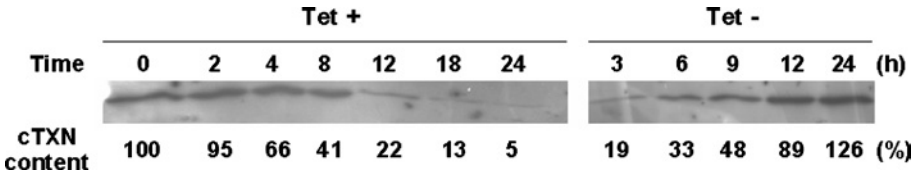

Figure 5 Western-blot analysis of short-term induction and withdrawal of cTXN down-regulation

A representative Western blot (clone C1) is shown for the time-dependent induction (Tet+) of cTXN knockdown and after withdrawal of tetracycline (Tet–). For the reversal of cTXN-RNAi, cells grown for 24 h in the presence of tetracycline were harvested by centrifugation, washed twice with medium without tetracycline and further cultured in this medium. 1×10^6 cells were applied per lane, and cTXN levels are expressed as percentage of the non-induced state (time point 0 for Tet+). Tet, tetracycline.

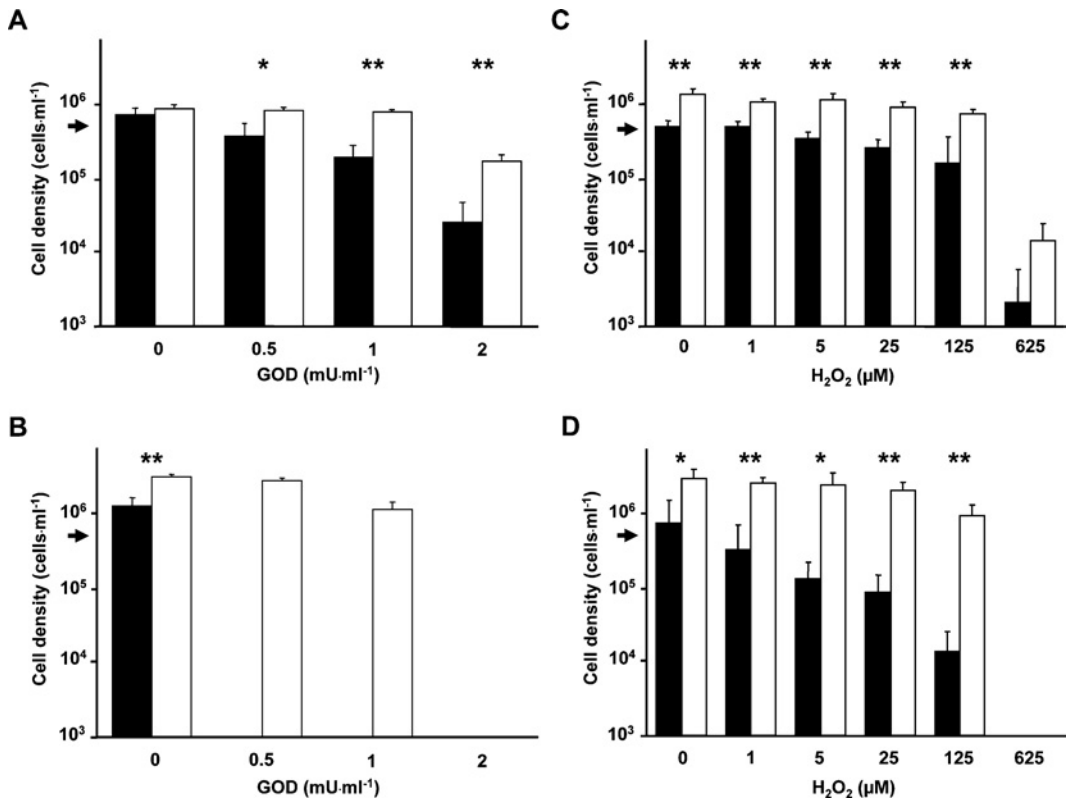

Figure 6 H_2O_2-sensitivity of cTXN-depleted bloodstream *T. brucei*

Susceptibility to H_2O_2 was studied in tetracycline-induced (black bars) and non-induced (white bars) cTXN-RNAi cell lines B1, B6 and D5; 5×10^5 cells · ml^{-1} were challenged with H_2O_2 generated by 0.5, 1 and 2 m-units · ml^{-1} GOD (**A**, **B**) or by adding 1, 5, 25, 125 and 625 μM H_2O_2 directly to the medium (**C**, **D**). After 6 h (**A**, **C**) and 24 h (**B**, **D**) exposure, cell viability was assessed by optical inspection under the light microscope. The results presented are the means for the averaged cell densities of the three clones with standard deviations. Asterisks indicate significant differences compared with non-induced controls, with $P < 0.05$ (*) or $P < 0.01$ (**) ($n = 3$; two-tailed Student's t test). The black arrow marks the initial cell density (5×10^5 cells · ml^{-1}).

the first series of experiments, oxidative stress was induced for 6 and 24 h in a medium without tetracycline and on cells in which cTXN-RNAi had been induced for 24 h. Under these conditions, cell viability did not significantly differ between cTXN-RNAi-induced and non-induced cultures (results not shown). We therefore determined the time required for RNAi-induced cells to resume expression of cTXN upon removal of tetracycline. As exemplified in Figure 5, 6–9 h after tetracycline withdrawal the concentration of cTXN was 33–48 % (~ 15 μM) of that in the non-induced cells (37 μM) and more than 10-fold higher than the lowest concentration obtained by RNAi (1 μM). Expression almost fully recovered 12 h after the withdrawal of tetracycline. In light of these results and in order to assure a sustained depletion of cTXN, the subsequent experiments were carried out in the presence of 1 μg · ml^{-1} tetracycline. The constant generation of H_2O_2 by 0.5 or 1 m-unit · ml^{-1} GOD had a significant effect on the growth of cTXN-depleted trypanosomes, but only marginally affected the control cultures (non-induced; Figure 6A). At 2 m-units · ml^{-1} GOD killed both the RNAi-induced and non-induced parasites, although the former cells were more sensitive than the controls. Incubation of the cultures for another 18 h with 0.5 and 1 m-unit · ml^{-1} of GOD killed cTXN-down-regulated trypanosomes, while non-induced cells were only arrested or even kept on growing (Figure 6B). Also using a bolus of H_2O_2, the TXN-depleted parasites showed a higher susceptibility (Figures 6C and 6D). The calculated LC_{50} (concentration of compound that is lethal to 50 % of the parasites under the test conditions) values for H_2O_2 after 6 h treatment were 32 and 144 μM for cTXN-RNAi-induced and non-induced parasites respectively. Thus depletion of cTXN increases the sensitivity of trypanosomes against H_2O_2 nearly 5-fold. At 24 h after the addition of H_2O_2, cTXN-depleted cells did not replicate any more and

the proportion of dead cells correlated with the concentration of H_2O_2 (Figure 6D). From these results, a 24 h LC_{50} of <1 μM H_2O_2 was estimated for the TXN-down-regulated parasites. Only the highest H_2O_2 concentration used (625 μM) completely killed the non-induced cultures, while lower concentrations impaired cell growth.

DISCUSSION

TXN has been proposed as a putative target molecule for the design of anti-trypanosomal agents because of low similarity to mammalian thioredoxins, its specificity for $T(SH)_2$, its function as a pleiotropic redox mediator and its participation in the rate-limiting step of the hydroperoxide metabolism of trypanosomatids [27]. As shown here, TXN is essential for bloodstream *T. brucei* and thus fulfils one of the prerequisites for a drug target. The failure of a previous RNAi study to discover a phenotype in cTXN-depleted parasites [18] is probably explained by the experimental system employed [21,22,25]. RNAi has become a valuable tool to investigate gene functionality in *T. brucei*, but leakage (production of dsRNA in absence of the inducer) or a reduced amount of dsRNA entering the RNAi pathway may thwart the efficiency of this method [28]. Testing of different host/vectors systems and screening of several clones appear to be mandatory to avoid a bias from clonal variations. Most importantly, in the case of essential gene products revertants are often observed within a very short time even with integrating vectors and the best host/vector combinations presently available. This narrows the time window for meaningful experiments, in particular if, as in the case of cTXN, a very abundant protein is slowly depleted and rapidly restored upon cessation of the RNAi. In accordance with the results of Wilkinson et al. [18], we could not observe any phenotype upon oxidative challenge, if dsRNA synthesis was not sustained by persistent presence of inducer.

Although we could efficiently deplete the cTXN mRNA in most of the transfectants, the cTXN concentration had to be lowered to 1 μM for approx. 48 h before growth and viability of bloodstream trypanosomes were significantly impaired. The delayed response to cTXN depletion may be surprising in view of the postulated role of the $T(SH)_2$/TXN couple as a key factor determining the intracellular redox milieu of the parasites [29]. However, the $T(SH)_2$-dependent reactions (Figure 1) can in principle proceed without the catalytic support of a TXN. In fact, $T(SH)_2$ and TXN are similar in terms of their redox potentials [midpoint potential (E_0') $= -249$ and -242 mV for TXN and $T(SH)_2$ respectively] and pK values (7.2 and 7.4 for TXN and $T(SH)_2$ respectively) [29]. Thus spontaneous reactions of $T(SH)_2$ with H_2O_2 [30], peroxynitrite [7] and even with ribonucleotide reductase [16] can occur provided that the concentration of the dithiol is sufficiently high. Thus, temporarily, $T(SH)_2$ may take over the functions of TXN. Indeed, as demonstrated here for the first time (Figure 4), the parasite can make use of this option by increasing the concentration of $T(SH)_2$ and the other low-molecular-mass thiols when it falls short of cTXN. A (partial) substitution of cTXN function by the trypanosomal thioredoxin (Trx) is not very likely. Although the latter protein catalyses most, if not all, TXN-dependent reactions, its cellular concentration is probably very low [31,32]. The crucial role of TXN in catalysing all these redox reactions is obvious from the (delayed) phenotype due to cTXN knockdown and from the lack of phenotype observed in Trx knockout cells [33]. Our results show that elevated concentrations of $T(SH)_2$ can, in the long term, not compensate for the cTXN-catalysed reactions. One can only speculate which of the cTXN-dependent reactions is finally responsible for the observed phenotype in the cTXN-depleted cells. Presumably it is the decrease in deoxyribonucleotides impairing DNA synthesis.

The importance of cTXN catalysis becomes even more obvious when the parasites are oxidatively challenged, i.e. under conditions where the spontaneous hydroperoxide detoxification competes with oxidative damage. A significantly decreased antioxidant capacity could clearly be demonstrated 30 h after initiation of cTXN depletion, i.e. at a time point at which still no phenotype was detectable in the unchallenged parasites. The TXN-dependent peroxidases catalyse the reduction of different hydroperoxide substrates with apparent second-order rate constants ranging from 1.2×10^4 to 1.7×10^7 $M^{-1} \cdot s^{-1}$ [6,8]. This implies that the peroxidase-catalysed reactions are by orders of magnitude faster than the spontaneous reactions of the hydroperoxides with $T(SH)_2$ (<1 to 10 $M^{-1} \cdot s^{-1}$) [30]. Without keeping the peroxidase(s) reduced by adequate concentrations of reduced cTXN, the hydroperoxides will have ample time to harm sensitive cellular targets. Interestingly, the concentration of cTXN is significantly higher in the parasite form infecting mammals than in that hosted by invertebrates. This difference could simply be attributed to the higher metabolic rate of bloodstream trypanosomes in comparison with procyclic forms [34], or more likely to the fact that bloodstream parasites have to cope with the innate immune response of the host.

Our results clearly demonstrate the essential role of cTXN and corroborate its importance in trypanosomal hydroperoxide metabolism. However, cTXN may not be an ideal drug target candidate. According to the data presented, its activity would have to be lowered to less than 5 % for a sustained period of time before a significant impairment of the parasites can be expected. This is clearly not an easy task with regard to the abundance of the protein. In addition, all thioredoxin-like proteins including TXN catalyse thiol–disulfide exchanges employing exposed cysteine residues, but do not have a classical hydrophobic active-site cleft. Also in the case of trypanothione reductase, which has been demonstrated to be indispensable by expression of a trans-dominant mutant and knockout studies [35,36], reduction of activity by more than 90 % was required to impair virulence of bloodstream *T. brucei* [36]. Thus the most upstream enzyme of the pathway, trypanothione synthetase, is a highly promising target molecule for the development of new antiparasitic drugs. The enzyme has been validated as drug target by means of inverse genetics in bloodstream [25] and procyclic forms of *T. brucei* [37]. Depletion of the mRNA of trypanothione synthetase in the parasite form infecting mammals lowered the level of $T(SH)_2$ by 85 %, which caused severe impairment of growth, viability and antioxidant defence [25]. As it is the case for all components of this pathway, the enzyme does not have a closely related counterpart in humans. In addition, its cellular concentration is low (0.005 % of the total soluble protein in *T. brucei*) [38]. Inhibition of trypanothione synthetase could also prevent any compensatory elevation of $T(SH)_2$ (see above), which might compromise the effect of inhibiting more downstream enzymes of the $T(SH)_2$ metabolism.

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