THE DITHIOL GLUTAREDOXINS OF AFRICAN TRYPANOSOMES HAVE DISTINCT ROLES AND ARE CLOSELY LINKED TO THE UNIQUE TRYPANOTHIONE METABOLISM

Sevgi Ceylan, Vera Seidel, Nicole Ziebart, Carsten Berndt, Natalie Dirdjaja, and R. Luise Krauth-Siegel

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA Constructs for RNA-Interference against Grx1 and Grx2 in T. brucei—With the pETtrx1b/*grx1* plasmid as template, two different fragments were amplified by PCR (94 °C for 2 min; 94 °C for 30 s; 56 °C for 30 s; 72 °C for 2 min; 30 cycles; 72 °C for 10 min). Amplification with the forward primer 5′–GAC AAG CTT ATG GAA CCC TCT ATC GCT TCG AT–3′ (the HindIII restriction site is underlined) and the reverse primer 5′–CA GAA TTC TGC AGT TAG CTC AGC AAA CCA TCG–3′ with EcoRI and PstI restriction sites resulted in a 291 bp insert which covered the coding region of *grx1*. The second PCR with the forward primer 5′–GAC GTT AAC ATG GAA CCC TCT ATC GCT TCG AT–3′, containing a HpaI restriction site, and the reverse primer 5′–CA GAA TTC TGC AG GCA GCC GGA TCT CAG TG–3′, with EcoRI and PstI sites, amplified a fragment of 370 bp which corresponded to the coding region plus additional 79 bp of the vector. The fragments were digested with the respective restriction enzymes and ligated into the pHD678 vector digested with HindIII and HpaI which resulted in pHD678ms/*ri-grx1* (ms, minus stuffer). The pHD678 vector contains a hygromycin resistence gene and allows the tet-inducible expression of the inserted fragment. Sure®2-Supercompetent cells (Stratagene, Amsterdam) were transformed and grown in LB medium containing 100 µg/ml carbenicillin. The plasmid was isolated. A NotI restriction site present in the vector-derived stretch was removed by PCR (94 °C for 2 min; 95 °C for 30 s; 55 °C for 1 min; 68 °C for 7 min; 18 cycles; 68 °C for 10 min) with the forward primer 5′-CGA CAA GCT TGC GGA CGC ACT CGA GCA CCA C-3′ and the reverse primer 5′-GTG GTG CTC GAG TGC GTC CGC AAG CTT GTC G-3′, using the Quick change® Site-Directed Mutagenesis Kit (Stratagene). The mutated base is underlined. *E. coli* XL1Blue cells were transformed and grown in LB medium containing 100 µg/ml carbenicillin. The plasmid DNA was isolated and the insert was sequenced in both directions. For the Grx2 RNA-interference construct the pETtrx1b/*grx2* plasmid served as template. Amplification with the forward primer 5′–GAC AAG CTT ATG GGA AAT AAC GCA TTG GAT C–3′ with HindIII site (underlined) and the reverse primer 5′-A GAA TTC TGC AGT CA TCC CCT TTC AAT TTC-3′ with EcoRI and PstI restriction sites resulted in a 327 bp fragment that corresponded to the coding region of *grx2*. Amplification with the forward primer 5′–GAC GTT AAC ATG GGA AAT AAC GCA TTG GAT C–3′ with a HpaI restriction site and the reverse primer used for the generation of the second *grx*1 fragment resulted in a 406 bp insert which covered the coding region plus 79 additional vector-derived bp. The pHD678ms/*ri-grx2* plasmid was obtained by exactly the same procedure described above for *grx1*.

DNA Constructs for the Overexpression of Grx1 and Grx2 in T. brucei—The coding region of *grx1* was amplified from genomic DNA using the forward Grx1 HindIII (5'GACAAGCTTATGCCCTCTATCGCTTCGAT3') and the reverse Grx1 BamHI (5'GCGGATCCGCTCAGCAAACCATCGAGTTTTC3') primers (restriction sites underlined). In the pETtrx1b/*grx2* plasmid (see above) a BamHI site was removed with forward (5'ATGGGAAATAACGCATTAGATCCGGCAAAGGCACCAC3') and reverse (5'GTGGTGCCTTTGCCGGATCTAATGCGTTATTTCCCAT3') primers and the QuickChange® II Site-Directed Mutagenesis Kit (mutated base underlined). The coding region of Grx2 was then amplified with forward Grx2 HindIII (5'GACAAGCTTATGAATAACGCATTAGATCCGG3') and reverse Grx2 BamHI (5'GCGGATCCGCCCCTTTCAATTTCGGCTGA3') primers removing the artificial N-terminal glycine codon (see above). The integrity of all constructs was verified by DNA sequencing. The constructs were cloned into the pHD1700 vector generating pHD1700/*grx1-c-myc*$_2$ and pHD1700/*grx2-c-myc*$_2$. The pHD1700 expression vector carries a hygromycin resistance cassette and allows a tet-inducible ectopic expression of a protein with C-terminal myc$_2$-tag.

SUPPLEMENTAL FIGURES

A

UAGAACAGUUUCUGUACUAUAUUG*AAUCAAGAAGAAACACAAACAUUUAGCACUUUCCCACCUCGUUUUU*
AUUCACUUACAAUACACCUUGAAGUGAAUAAAACUAAGGUUACUCUGUAGAGUGGGACAAAAACUUUGUU
AUAUAUAUAUAUAUAUAUUUAUUUAUUUAUUUAUCUUGUAUUUUGUCCCCAGCACGUGGUAUCUGACUGA
*UCAAUUACAUC***AUG**CCCUCUAUCGCUUCGAUGAUUAAAGGGAAUAAAGUGGUUGUUUUCUCAUGGGUCAC**
UUGCCCCUACUGCGUCCGAGCAGAGAAGUUACUUCACGCACGCACUAAGGAUAUUACCGUACAUUAUGUG
GAUAAGAUGAGCGAAGGAGAACAACUGCGCGGUGAGAUUUACCAAGCAUACAAACACGAAACGGUUCCCG
CCAUAUUUAUUAAUGGAAAUUUCAUUGGCGGUUGCAGCGAUUUGGAGGCCCUUGACAAAGAGGGAAAACU
CGAUGGUUUGCUGAGCUAA*AUUUGAGACAGUGAGGAGGAUUUUUUUAAAAAAAAAAAGAAGAGAAAAAAG*
AGAAAGGGGGGAUUACGUGAAUAUAUAUGCGGUUGCAACCCUCUGAUUAAUAUAGUGCCAAACGAGAGGU
GUUUGCAUAUGUUGAGGGGGUAAACUACACACCCUUCCUUUAUAUACAUCUCCUUCAUCCUUCACUUGAU
CGCUUUCAUUUUUUUUAUAUUUUUGUUCCUGCUUAUAUUAGGGGUUAUAAUGCAUUAUGCAUGAUGUGUU
CUGCUCAUUUCAUACACGAGGGAAGGGAAAACGGAAACGGAAAUGCAAGAACGGGAGAGAGUAGGAGCGA
GUGAACUGACAUCAAUUCAAUCGGGGGAAGUGUUUAAAAACACCUCACCGGUAUAACUUCCCCUAUUGAU
*ACGUUACGCACGACGCUAUUUCUGUUGUUG****AAAAAAAAAAAAAn***

B

UAGAACAGUUUCUGUACUAUAUUG*GUAGAAUUUUUUUUUAAUAAAAAUAGUAAAAGGAACUACUAAUAACG*
GAGUUCCAAUCCUUUUUGGCCAGCUUUAACGACACUUCUGCAUAAGGGGAGGGGGGAAGUUAUUUUUAAA
AAAAAAAGUAAAGAAGUUGUGGAAUUCAAGUGCGCUGGUAUCAAAUACAUAAGUCACGUGAUUGCGUCCA
AAGAAAACCAGUGAAGCUGGAUUUAUUAACGUCAGGCAGUUCCAAACAAACACAAAUAAGAUUUAGUAUA
AGGUUACACAUAUUUAGUUGUUGAUUUACAUAUAUAGUUGACUUGUUUGCUUUAUUUUGUUCUAUUUUAU
*UUUAUUCUAUUUUUUUCUUUGUACGUUGCUGUGAGGGACCUACCAGG***AUG**AAUAACGCAUUGGAUCCGGC**
AAAGGCACCACAAUUUCUUGAUAUGAUGUUGCGCCGCAAUAAAAUGGUAAUGGUGUCCGCCACGUAUCGU
CAGUUCUGCACAAAACUGAAGAUGUUACUGAUUGAACUCAAACACCGUUUCGUUUCACUUGAAAUUGAUA
UUAUUCCCAACGGUCGUGAAGUGUUUGCAGAAGUGGUGGGACGCACGGGUGUACAUACGGUGCCACAAGU
UUUUCUUAAUGGGAAGUAUUUUGGCGGUUAUGAUGAACUUGUUGCAAUGUACCGGGCAGGGCACCUUUCA
GCCGAAAUUGAAAGGGGAUGA*AGAGGUAAUUAUUGUUUAUGUAUUGUGAGCAUAGGCGUAACGAAGUAUA*
UUUAUGUGUAUGUUCACUUCGAUGGAGCGUACAGCUGUAAUGUUUAAUUGUCUCAUUUUGAGACGGGCAG
UGGUCAUUUGGUUUUUUUUGUUGUUAUUGUUGUUGUUGUUUUUUUUUCUUAUGUUUUUUUUUGUUUCAUC
UGUCACCACUUUCUUUCUUUCUUUCUUUCUUCGUUCCAUAGAAAUUUGAACGAUGAUGUGGAAAGGGAGG
AUUGUGUGGAGAAAAUGAGGCACACGAACAUGGAAGUUUGUUAAUAGGUAUUCAUACUCACACAUGUAUU
UGUAUGGCGUGCACAUCUCGCUGAUUCUCUGUUUUGGUUUUAUUUGUUGUUGUAUGCUGCUCGGCAGGGG
*AAGGGAG****AAAAAAAAAAAAAn***

FIGURE S1. mRNA Sequences of *T. brucei* Grx1 and Grx2. The mRNA sequences were obtained by PCR on cDNA from procyclic *T. brucei*. *A*, a Grx1-specific forward primer (5′ C ATG CCC TCT ATC GCT TCG 3′) and a poly T primer (5′GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TTN) resulted in a 434 bp 3′untranslated region (UTR). PCR with a *spliced leader* primer (5′TAG AAC AGT TTC TGT ACT ATA TTG 3`) and a Grx1-specific reverse primer (5′CGT TTC GTG TTT GTA TGC TTG 3′) yielded a 197 bp 5′UTR. *B*, The untranslated regions of Grx2 were obtained with a gene specific forward primer (5′GG ATG AAT AAC GCA TTG GAT 3′) and the poly T primer resulting in a 409 bp 3′UTR, and with the *spliced leader* primer and a gene specific reverse primer (5′CTT CTG CAA ACA CTT CAC GAC 3′) yielding a 373 bp 5′UTR. The PCR products were sequenced in both directions. The 5′and 3′ UTRs are shown in italics, the coding regions are given in bold letters with the start and stop codons being underlined. The sequences of the *spliced leader* and poly T primers are depicted in italic bold letters.

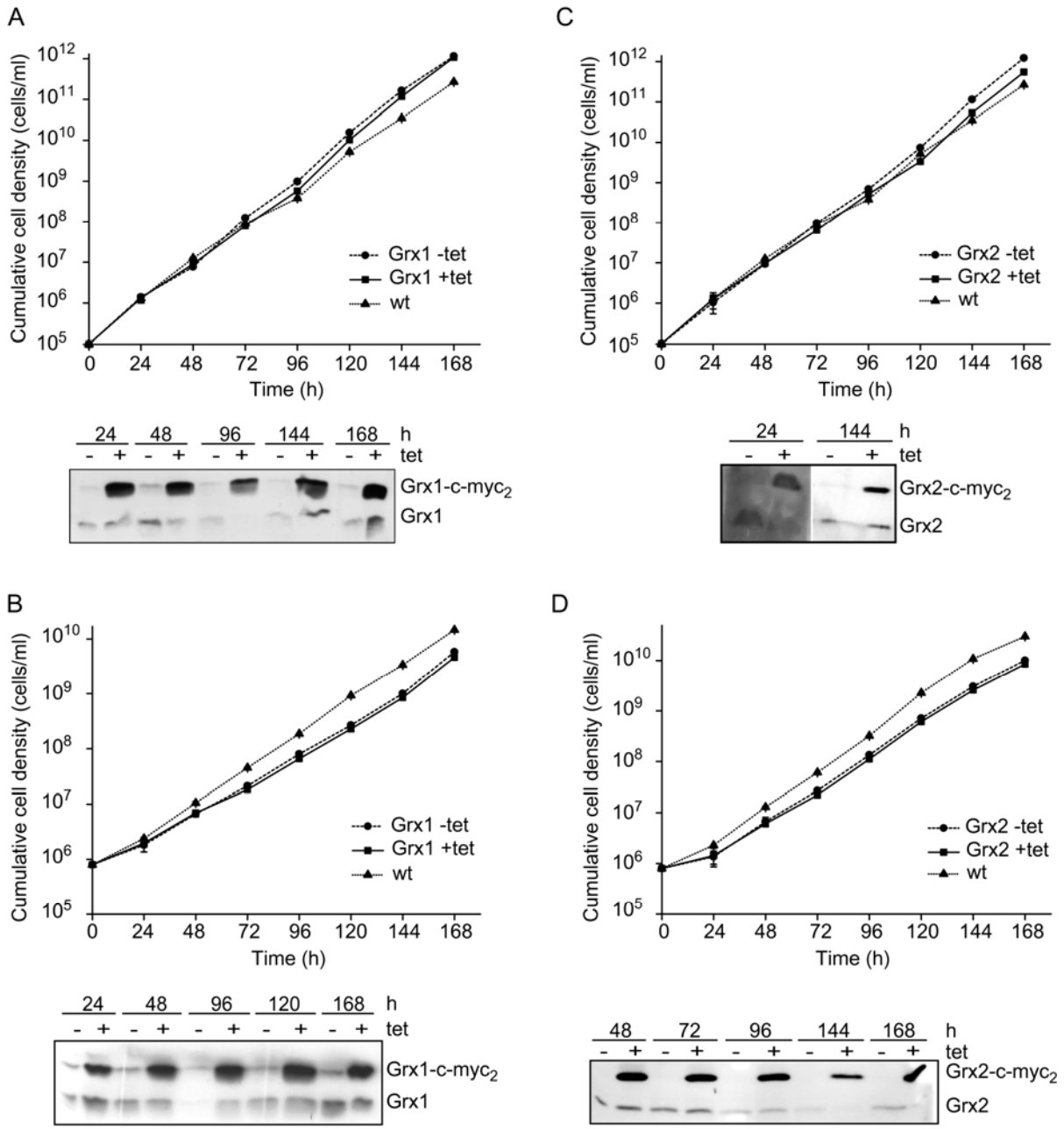

FIGURE S2. Growth curves and protein levels of *T. brucei* cell lines overexpressing Grx1 or Grx2. *A*, bloodstream and *B*, procyclic cells transfected with pHD1700/*grx1-c-myc*$_2$ and *C*, bloodstream and *D*, procyclic cells transfected with pHD1700/*grx2-c-myc*$_2$ were grown in the presence (+ tet) and absence (- tet) of tetracycline. Every 24 h, the cells were counted, samples were withdrawn for Western blot analysis, and the cultures were diluted with fresh medium (+/- tet) to the initial cell density. Shown is the mean ± SD of the cumulative cell densities of three clones. 3 x 10^6 bloodstream and 1 x 10^7 procyclic cells per lane were subjected to Western blot analysis with anti-Grx1 and anti-Grx2 antibodies. The blots are representative for the three clones analyzed in each case.

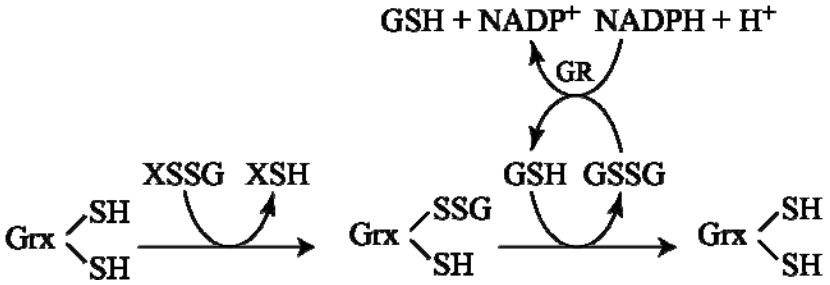

FIGURE S3. Reaction scheme for the reduction of GSH-mixed disulfides by Grxs. XSSG is a mixed disulfide between a low molecular mass or protein thiol (XSH) and glutathione; GR, glutathione reductase.

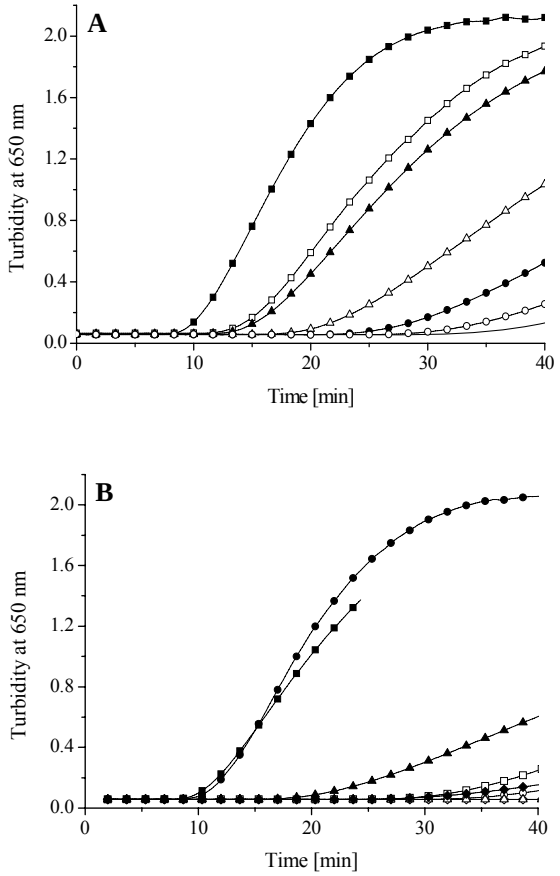

FIGURE S4. Grx-catalyzed reduction of insulin. The reaction mixtures contained in 100 mM potassium phosphate, 2 mM EDTA, pH 7.0, 130 μM insulin, 1.2 mM thiol and varying concentrations of the respective Grx. The increase in turbidity is plotted against the reaction time. *A*, in the presence of 663 μM DTE. −, control lacking Grx; ■, 5 μM and □ 2.5 μM *E. coli* Grx1; ▲, 22 μM and Δ, 8 μM Grx2, ●, 43 μM and ○, 14 μM Grx1. *B*, *E. coli* Grx1-catalyzed reduction by different thiols. □ and ■, 624 μM $T(SH)_2$; Δ and ▲, 1.25 mM Gsp; ○ and ●, 628 μM DTE; ◊ and ♦, 1.3 mM GSH. Empty and filled symbols display the reactions in the absence and presence of 5 μM *E. coli* Grx1, respectively.

SUPPLEMENTAL TABLES

Supplemental Table S1

HED reductase activity of different Grxs

T. brucei Grx1 and Grx2 as well as *E. coli* Grx1 were subjected to standard HED assays at pH 8.0 with 0.8 mM HED and 1 mM GSH as described under "Experimental Procedures". The activities were plotted against the Grx concentration, the slope of the linear regression line corresponding to the specific activity. In the case of Grx1 and *E. coli* Grx1, the data presented are the mean ± standard deviations of four independent series of measurements. For Grx2, the values were derived from a duplicate measurement of five different concentrations.

	Specific activity
	[U/mg]
T. brucei Grx1	26.1 ± 4.4
T. brucei Grx2	3.2 ± 0.2
E. coli Grx1	100 ± 19
E. coli Grx1[a]	156.8 ± 13.5
Human Grx1[a]	138.7 ± 21.8
Human Grx2[a]	16.0 ± 2.8

[a]Porras, P., Pedrajas, J. R., Martinez-Galisteo, E., Padilla, C. A., Johansson, C., Holmgren, A., and Barcena, J. A. (2002) *Biochem. Biophys. Res. Commun.* **295**, 1046-1051