Overexpression of a Cytochrome $b_5$ Reductase-like Protein Causes Kinetoplast DNA Loss in *Trypanosoma brucei*

Received for publication, March 27, 2006. Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.M602880200

Shawn A. Motyka, Mark E. Drew, Gökkben Yildirir, and Paul T. Englund

From the Department of Biological Chemistry, Johns Hopkins School of Medicine, Baltimore, Maryland 21205

The mitochondrial genome of trypanosomes, termed kinetoplast DNA (kDNA), contains thousands of minicircles and dozens of maxicircles topologically interlocked in a network. To identify proteins involved in network replication, we screened an inducible RNA interference-based genomic library for cells that lose kinetoplast DNA. In one cloned cell line with inducible kinetoplast DNA loss, we found that the RNA interference vector had aberrantly integrated into the genome resulting in overexpression of genes downstream of the integration site (Motyka, S. A., Zhao, Z., Gull, K., and Englund, P. T. (2004) *Mol. Biochem. Parasitol.* 134, 163–167). We now report that the relevant overexpressed gene encodes a mitochondrial cytochrome $b_5$ reductase-like protein. This overexpression caused kDNA loss by oxidation/inactivation of the universal minicircle sequence-binding protein, which normally binds the minicircle replication origin and triggers replication.

The rapid loss of maxicircles suggests that the universal minicircle sequence-binding protein might also control maxicircle replication. Several lines of evidence indicate that the cytochrome $b_5$ reductase-like protein controls the oxidation status of the universal minicircle sequence-binding protein via tryparedoxin, a mitochondrial redox protein. For example, overexpression of mitochondrial tryparedoxin peroxidase, which utilizes tryparedoxin, also caused oxidation of the universal minicircle sequence-binding protein and kDNA loss. Furthermore, the growth defect caused by overexpression of cytochrome $b_5$ reductase-like protein could be partially rescued by simultaneously overexpressing tryparedoxin.

*Trypanosoma brucei* is a flagellated protozoan parasite that causes sleeping sickness in humans and related disease in cattle. Trypanosomes are early branching eukaryotes and one of the first to contain a mitochondrion (1). The single mitochondrion is a tubular structure, which contains an unusual DNA termed “kinetoplast DNA” (kDNA). kDNA is a planar network composed of several thousand topologically interlocked DNA circles. Within the mitochondrial matrix the network is condensed into a disk-shaped structure that is positioned near the flagellar basal body. The network contains two types of circles known as maxicircles and minicircles. Maxicircles (23 kb each) encode the many guide RNAs that are templates for editing (see Refs. 6 and 7.)

Although roughly 30 proteins have been identified that likely participate in kDNA replication or maintenance, we speculate based on the complexity of the process that more than 100 proteins might be involved (6). To identify new proteins we developed a forward genetic approach using an RNA interference (RNAi)-based library. We created the library by ligating fragments of *T. brucei* genomic DNA into the tetracycline (tet)-inducible RNAi vector pZJM (8). We previously used the library to screen for trypanosomes with various phenotypes and then identified the relevant gene by PCR and sequencing of the pZJM insert (9, 10). To identify genes involved in kDNA replication, we screened for kDNA loss, a phenotype commonly observed after RNAi knockdown of replication proteins (4, 11, 12). Since kDNA loss is lethal, it was necessary to clone cells from the library prior to inducing RNAi. In one candidate clone, in which tet induction caused kDNA loss, we discovered that the RNAi vector had not properly integrated into the rDNA spacer region (13). This aberrant integration, into the gene targeted by the pZJM insert, caused the dual T7 promoters of the vector to be directed upstream and downstream from the integration site. Consequently, tet induction led to overexpression of 10 genes downstream of one of the T7 promoters. Antisense RNA was potentially transcribed from the other T7 promoter, but we did not detect silencing of upstream genes (13). Therefore, we presumed that overexpression of one of the downstream genes was responsible for kDNA loss. We now report the identification of this gene, cytochrome $b_5$ reductase-like (CBRL), and characterize the mechanism by which its overexpression confers loss of kDNA.
A Trypanosome Cytochrome b$_5$ Reductase-like Protein

EXPERIMENTAL PROCEDURES

Trypanosome Growth and Transfection—All experiments were performed using the T. brucei procyclic 29-13 cell line (the generous gift of Drs. Elizabeth Wirtz and George Cross), which constitutively expresses both T7 RNA polymerase and the tet repressor (14–16). Cells were grown in SDM-79 medium (17) supplemented with 10% fetal bovine serum and the appropriate antibiotics (15 µg/ml G418, 50 µg/ml hygromycin, 2.5 µg/ml phleomycin, and 10 µg/ml blasticidin) at 28 °C under 5% CO$_2$. Fetal bovine serum at 15% was used in medium for cloning by limiting dilution. Cells were transfected by electroporation in Cytomix (18), and growth was monitored with a Coulter counter (model Z1, Coulter Corp.).

Overexpression and Localization—For overexpression, we cloned the open reading frame of the gene of interest downstream of a tet-regulated T7 promoter in the pLew111 vector (the generous gift of G. Cross, unpublished, see tryps.rockefeller.edu for description of pLew111) that we modified by inserting an unregulated T7 promoter sequence (5’-TAATAC-GACCTCACTATAG) immediately upstream of the drug resistance marker (ble) to make pLew111(2T7) (diagrammed in Fig. 1A). The additional T7 promoter eliminates the need to add tet to the culture medium during selection, a beneficial feature if the overexpressed protein is toxic. Overexpression was induced with 1 µg/ml tet, which was added every 2 days even if the cells were not diluted.

For intracellular localization, we cloned the CBRL open reading frame (minus the stop codon) into a modified version of pLew111(2T7). The multicloning site was expanded to include MluI, XbaI, XhoI, and an Spel site. The multicloning site was expanded to include MluI, XbaI, XhoI, and a Spel site. The sequence coding for the green fluorescent protein (GFP) was inserted downstream of the multicloning site, creating pLew111(2T7)GFP (diagrammed in Fig. 2A). Expression of the GFP fusion protein was induced with tet for 24–48 h, and live cells were visualized using a Zeiss Axioskop microscope. Images were captured with a Retiga Exi charge-coupled device camera (QImaging) and analyzed using IP Lab software (Scion). For co-localization, live cells were stained with 40 nM MitoTracker Red (Molecular Probes) for 20 min and washed twice with PBS prior to mounting.

RNAi and Knock-out of CBRL—For RNAi, a 500-bp fragment of the CBRL open reading frame (starting at 5’-TCCGGCTGC) was cloned into the pZIM vector. Inducible RNAi was performed as previously described (8). For knock-out via allelic replacement, 500 bp from the CBRL 5’ (amplified using primer pair KO5F 5’-ATGGCAGCCTCAGCCGTCGTC and KO5R 5’-ATGGGCACCTTTTCCAAGC) and 3’ (amplified using primer pair KO3F 5’-CTCGTTTTCTACGGGAGGAA and KO3R 5’-CTGTTGCGGTGGTGTGTTT) untranslated regions were cloned on opposite sides of a drug resistance cassette in the pKO vector (a generous gift of Dr. James Bangs) (19). The cassettes (containing either the blasticidin or puromycin resistance marker) were then sequentially transfected into 29-13 procyclic trypanosomes to allow for selection of stable lines prior to transfection with the second marker. For unknown reasons, selecting for puromycin resistance before blasticidin resistance was more efficient than the reverse order for generating double knock-out parasites.

Northern Blotting, Southern Blotting, and Hybridization—For Northern blotting, RNA was isolated using the Purescript RNA isolation kit (Gentra Systems). RNA from 1.25 × 10$^6$ parasites was loaded per lane and fractionated on a 1.5% agarose gel containing 7% formaldehyde. RNA was then transferred to a GeneScreen Plus membrane (PerkinElmer Life Sciences). Hybridization and washing conditions were as described previously (8).

Total DNA for Southern blotting was isolated using a genomic DNA isolation kit (Gentra Systems). For gene knock-out analysis, genomic DNA (1 µg) was digested with Xhol and HindIII (5 units/µg of DNA) for 20 h at 37 °C and fractionated on a 0.7% agarose gel with the buffer containing 1 µg/ml ethidium bromide. The DNA was partly depurinated by soaking the gel in 0.25 M HCl for 15 min, rinsing three times with water, and treating with 0.4 M NaOH/1.5 M NaCl for 15 min before transfer to GeneScreen Plus membrane and hybridization (20). Blots were hybridized with appropriate 32P-labeled probes made with a random primer DNA-labeling system (Invitrogen). Quantitation of total mini- and maxicircles and of free minicircles by phosphorimaging was as previously described (4).

Western Blotting—Mid-log phase cells (5 × 10$^6$ cells/ml) were centrifuged (2,500 × g, 3 min) and resuspended (2 × 10$^6$ cells/lane) in 10 µl of non-reducing sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, bromophenol blue) or reducing sample buffer (same as non-reducing except with 10% β-mercaptoethanol). After boiling for 5 min, samples were fractionated by SDS-PAGE (12.5% acrylamide) and transferred to a polyvinylidene difluoride membrane for probing. Universal minicircle sequence-binding protein (UMSBP) was detected using an affinity-purified rabbit polyclonal antibody (diluted 1:1,000) against the full-length Crithidia fasciculata UMSBP (a gift of Dr. Joseph Shlomai). RNA-editing proteins were detected using rabbit polyclonal antibodies (a gift of Dr. Barbara Sollner-Webb) against Band II/TbMP81 (1:500), Band III/TbMP63 (1:2,000), or Band VI/TbMP52 (1:1,000) (21). The BB2 epitope (EVHTNQDPLD) was detected using a mouse monoclonal antibody (1:100, a gift of Dr. Chris Tschudi) (22, 23). Herporadish peroxidase-conjugated goat anti-rabbit (diluted 1:10,000) or goat antimouse (diluted 1:20,000) secondary antibody (Pierce) was detected by chemiluminescence using ECL Western blotting reagents (Amersham Biosciences).

RESULTS

Identification of the Gene Responsible for kDNA Loss—As described in the introduction, aberrant integration of our RNAi vector led to overexpression of 10 genes downstream of the integration site and to loss of kDNA. To identify the gene responsible for kDNA loss, we cloned each of the 10 open reading frames separately into a tet-inducible overexpression vector (Fig. 1A). We found that overexpression of one of these genes, whose product is homologous to cytochrome b$_5$ reductase, led to kDNA loss (to be described below). We designate this protein “CBRL,” for cytochrome b$_5$ reductase-like (GenBank$^\text{TM}$ accession number 71755709). A Northern blot confirmed that tet induction caused a massive increase in CBRL mRNA (Fig. 1B, compare day 0 with days 1–3). Note that there is a modest overexpression at day 0 relative to the level in wild type, indicating that there is “leaky” overexpression in the absence of tet. We attempted to produce antibodies in rat against recombinant CBRL, but our best preparation was not satisfactory for probing a Western blot. Therefore, to assess CBRL overexpression we tagged it at its N terminus with the 10-amino acid BB2 epitope (22, 23). We overexpressed the fusion protein from pLew111(2T7), and a Western blot revealed a significant accumulation of epitope-tagged CBRL (Fig. 1C). Overexpression of either native (Fig. 1D) or epitope-tagged (not shown) CBRL caused a rapid slowing of the growth of the parasite.

As mentioned above, CBRL resembles cytochrome b$_5$ reductase (b$_5$R), a eukaryotic redox protein that is anchored to mitochondrial or ER membranes via an N-terminal hydrophobic tail (24). The catalytic C-terminal domain of b$_5$R (~300 amino acids) contains a non-covalently bound FAD and has an NADH binding site. This domain is positioned in the cytosol, where it passes reducing equivalents from NADH to cytochrome b$_5$ for use by enzymes such as stearoyl-CoA desaturase (for review, see Ref. 25). The domain structure of CBRL is similar to that of b$_5$R except that CBRL has an additional N-terminal
A Trypanosome Cytochrome b<sub>5</sub> Reductase-like Protein

66-amino acid extension that lacks homology to any other sequence in the GenBank™ non-redundant data base (except for apparent orthologs in the related parasites Trypanosoma cruzi and Leishmania major). The 302-residue C terminus of CBRL contains no recognizable domains except for poorly conserved FAD and NADH binding sites.

Intracellular Localization of CBRL—We localized a CBRL-GFP fusion protein expressed from a tet-inducible overexpression vector (Fig. 2A). The fluorescence was concentrated in a tubular organelle with a staining pattern similar to that reported for other mitochondrial proteins (11, 26, 27). Co-localization of CBRL-GFP (Fig. 2B, left panels) with MitoTracker Red (Fig. 2B, right panels) confirmed that this CBRL is mitochondrial. Because the CBRL-GFP in this experiment was overexpressed, there was a shrinking and loss of kDNA comparable to that produced by overexpression of native CBRL (see below).

CBRL Is Not Essential for Growth—We next tested whether CBRL itself is required for cell growth. In an inducible RNAi experiment using the pZJM vector, there was >90% reduction in CBRL mRNA after 2 days, but we observed no effect on cell growth in our standard culture medium (data not shown). To address this issue more rigorously, we replaced both alleles of the CBRL gene with drug resistance genes by homologous recombination. Although a Southern blot confirmed that the CBRL gene was deleted from the genome (Fig. 3A) and a Northern blot detected no mRNA (Fig. 3B), the cells grew normally (Fig. 3C). Also, there was no effect on kDNA detectable by fluorescence microscopy of DAPI-stained cells (data not shown). Therefore, although CBRL overexpression caused kDNA loss and growth inhibition, its absence appeared to have no deleterious effects at least under the culture conditions used.

Loss of kDNA Networks following CBRL Overexpression—We induced CBRL overexpression and then followed the status of kDNA by visual analysis of DAPI-stained cells. As shown in Fig. 4 (A and B), shrinking and loss of kDNA networks proceeded slowly until day 9 when ~31% of the cells had small kinetoplasts, and 63% had none at all. After day 9, cells with normal-size kDNA began to appear in larger numbers, and cells no longer overexpressed CBRL mRNA (data not shown; note also, in Fig. 1D, that recovery of cell growth began by about day 13). The shrinking and loss of kDNA suggested that CBRL overexpression affects kDNA replication or maintenance.

We also monitored kDNA loss by Southern blot. We isolated total DNA at various times during the course of CBRL overexpression, digested it with HindIII and XbaI, fractionated the digests on an agarose gel, and transferred the fragments to a membrane. The total maxicircle level, determined with a probe for the 1.4-kb XbaI fragment (Fig. 5A, upper panel), rapidly decreased for 4 days following CBRL overexpression to ~20% of the day 0 level (Fig. 5B). After day 4 the maxicircle level remained relatively constant. Probing the same gel for the heterogenous minicircle population revealed different kinetics. Focused on singly and multiply cleaved fragments between 0.5 and 1.0 kb (4) (Fig. 5C), the CBRL mRNA appeared as a doublet. The less abundant component (*), also present in wild-type cells, is the transcript produced from the normal genomic locus. The more abundant component (**) corresponds to the transcript produced from the ectopic copy, which does not use the CBRL genomic 5' and 3' untranslated sequences. Longer exposure revealed a band just above 2.4 kb at days 1–3 that could be an mRNA precursor. The blot was stripped and reprobed for tubulin mRNA as a load control (lower panel). C, Western blot of total protein from cells uninduced (U) or induced (I) for 2 days to overexpress BB2 epitope-tagged CBRL. The blot was probed for BB2, and the fusion protein is denoted by **. The background signal serves as an internal load control. The signal from wild-type cells was similar to that of uninduced cells (not shown). D, growth curve of cells either uninduced or induced for CBRL overexpression. Plotted values are a product of measured values and the dilution factor. Uninduced cells grew at the same rate as wild-type cells, indicating that the leaky CBRL mRNA expression observed in the Northern blot (day 0) does not affect cell growth. Cells recover growth around day 13, and at that time CBRL mRNA levels revert to approximately normal (not shown).
possibility that the kDNA network may have fragmented or decatenated as a result of CBRL overexpression.

**Effect of CBRL Overexpression on the Free Minicircle Population**

Minicircles are released from the network as individual covalently closed circles, and these free minicircles then replicate unidirectionally as theta structures. Replication results in production of two daughter circles, containing one or multiple gaps, which are ultimately reattached to the network. To determine if CBRL overexpression perturbed this pathway, we evaluated the free minicircle population by Southern blot (Fig. 6A). Because kDNA networks underwent shrinking and loss (Fig. 4), whereas a majority of minicircles persisted (Fig. 5), we anticipated an increase in free minicircles or network fragments as a function of time of CBRL overexpression. In fact there was a continuous rise in the level of covalently closed free minicircles (Fig. 6B), and after day 2 there was also appearance of catenated minicircle oligomers (species migrating above the level indicated by the two asterisks) as well as covalently closed dimers. The latter species co-migrates with gapped/nicked minicircle monomers on the one-dimensional gel (Fig. 6A) but was resolved in a second dimension run in 30 mM NaOH (data not shown). A possible explanation of the results in Figs. 5 and 6 is that covalently closed minicircles are released from the network during CBRL overexpression but are inhibited in their ability to initiate replication.

**Overexpression of CBRL Causes Oligomerization of UMSBP**

How can CBRL overexpression cause these effects on kDNA? If CBRL is a redox protein (as suggested by its candidate NADH and FAD binding sites), it may act through UMSBP, a molecule that binds the minicircle replication origin and presumably triggers replication initiation. There is recent evidence that *C. fasciculata* UMSBP may be regulated by redox (28). UMSBP has five CCHC-type zinc knuckles, and it can be oligomerized by oxidation of cysteines in the zinc knuckles to form inactive disulfide-linked multimers. Reduction of the multimer cleaves the disulfide bridge, allows regeneration of the zinc knuckles, and converts UMSBP into an active monomeric protein that binds the replication origin (28). Whereas *C. fasciculata* probably...
has only a single species of UMSBP. * has two isoforms, one with 5 zinc knuckles (15 kDa) and one with 7 (21 kDa). The * isoforms share 60% identity over their five shared zinc knuckles, and each is ~38% identical to the * ortholog. It is not known why * apparently has two UMSBPs.

To test whether CBRL overexpression affects the oxidation state of UMSBP, we used a Western blot to analyze the oligomerization status of UMSBP in uninduced cells and cells overexpressing CBRL. We fractionated the proteins by non-reducing or reducing SDS-PAGE and detected the * UMSBP species with an antibody against * UMSBP. Under non-reducing conditions, we detected the two UMSBP isoforms in both wild-type and uninduced cells (Fig. 7, left panel, marked by single and double asterisks). However, upon CBRL overexpression for 3 days, there was a decrease of both isoforms of monomeric UMSBP and the appearance of species with higher molecular masses (see proteins at 60 and 80 kDa in the smear just below the slot), which likely represent oligomerized UMSBP (lane I in Fig. 7, left panel). The appearance of oligomerized UMSBP was reproducible and could be detected as early as 1 day after CBRL overexpression. Under reducing conditions, UMSBP is mostly monomeric in wild-type, uninduced, and induced cells indicating that oligomerization is mediated by disulfide bonds (Fig. 7, right panel). The proteins at ~60 and ~80 kDa in the reducing gel (also observed in a similar experiment in Fig. 8) could be either cross-reacting proteins or UMSBP species that are not subject to reduction. In control experiments, we found using Western blots that three other mitochondrial zinc finger-containing proteins (the RNA-editing proteins band II/TbMP81, band III/TbMP63, and band VI/TbMP52 (21)) did not oligomerize when CBRL was overexpressed (data not shown). Below we address the question of how CBRL overexpression affects the redox state of UMSBP.
A Trypanosome Cytochrome \( b_5 \) Reductase-like Protein

**A** Trypanosome Cytochrome \( b_5 \) Reductase-like Protein

**FIGURE 8.** Effect of overexpression of mitochondrial tryparedoxin peroxidase on kDNA and UMSBP. A, cells were induced to overexpress tryparedoxin peroxidase for 3 days and stained with DAPI. kDNA size indicated as in Fig. 4A, B, Western blot of total protein in cells uninduced (day 0) or induced (days 1 and 3) to overexpress tryparedoxin peroxidase. Proteins were fractionated by non-reducing (left panel) or reducing (right panel) SDS-PAGE, and blotted for UMSBP as in Fig. 7.

Does Loss of Reduced Tryparedoxin Mediate UMSBP Oligomerization?—We next considered the possibility that the link between overexpression of CBRL and UMSBP oligomerization could be the redox protein tryparedoxin. In an *in vitro* reaction, reduced tryparedoxin can convert oligomeric and inactive UMSBP into an active monomeric form that binds the minicircle replication origin (28). Overexpression of CBRL might deplete reduced tryparedoxin, allowing the oxidation of UMSBP.

Tryparedoxin is in the thioredoxin superfamily. It is maintained in its reduced state by trypanothione (N1,N8-bis(glutathionyl)permidine), the major low molecular weight reductant in trypanosomes. There is both a cytosolic form of tryparedoxin and a second form postulated to be mitochondrial (29). Unlike cytosolic tryparedoxin, the putative mitochondrial tryparedoxin has a hydrophobic sequence (as determined by a hydrophy plot) that could anchor it to the mitochondrial membrane where it might interact with and be oxidized by CBRL. We made exhaustive attempts to express in recombinant form the C-terminal domain of CBRL for redox activity in an assay containing mitochondrial tryparedoxin and UMSBP. Unfortunately, we were unable to produce the CBRL C-terminal domain as a soluble protein in either *E. coli* or Pichia pastoris.

We therefore turned to another redox protein, mitochondrial tryparedoxin peroxidase, to evaluate the effects of tryparedoxin on UMSBP. It is well documented that tryparedoxin peroxidase uses reduced tryparedoxin to reduce hydroperoxides (see Fig. 10 below) (30). Therefore we tested whether overexpression of tryparedoxin peroxidase could deplete the mitochondrion of reduced tryparedoxin, thus allowing UMSBP to oligomerize. We induced overexpression of mitochondrial tryparedoxin peroxidase and found, as with CBRL, both kDNA shrinkage or loss (Fig. 8A) and UMSBP oligomerization (Fig. 8B). As a control, we overexpressed eight other predicted mitochondrial proteins (four DNA helases, the two UMSBP isoforms, mitochondrial tryparedoxin, and mitochondrial HSP70) and found that none caused either kDNA loss or UMSBP oligomerization (data not shown). These results implicated tryparedoxin as a factor in controlling UMSBP oligomerization.

**Co-overexpression of Mitochondrial Tryparedoxin Rescues Cells Overexpressing CBRL**—If overexpression of CBRL or tryparedoxin peroxidase depletes the mitochondrion of reduced tryparedoxin, it might be possible to rescue the cells by co-overexpressing tryparedoxin. We therefore cloned the open reading frame of the putative mitochondrial tryparedoxin into the pXSGFP3MFSU vector (31), adding a stop codon prior to the GFP coding sequence. Overexpression from this vector is constitutive, utilizing a trypanosome procyclin promoter. We then transfected this construct into cells containing a construct for inducible overexpression of CBRL. As a control, we did a similar experiment with constitutive overexpression of cytosolic tryparedoxin. We found that uninduced cells grew at a rate similar to that of untransfected cells, indicating that constitutive overexpression of either tryparedoxin did not substantially affect cell growth (Fig. 9A). Upon CBRL overexpression, however, cells expressing cytosolic tryparedoxin stopped growing after 1 day and lost kDNA, similar to the parental cells, which overexpress CBRL alone. In contrast, cells expressing mitochondrial tryparedoxin continued to propagate upon CBRL overexpression, although the doubling time was 50% greater than for uninduced cells (Fig. 9A). A Northern blot revealed that both cell lines massively overexpressed CBRL mRNA upon tet induction (Fig. 9B). These results indicate that the effect of CBRL overexpression can be partly reversed by simultaneously overexpressing mitochondrial tryparedoxin.

**DISCUSSION**

To identify new kDNA replication proteins, we used a forward genetic screen of an RNAi library. In one clone with tet-inducible kDNA loss, there was aberrant integration of the RNAi vector causing overexpression of genes downstream of the integration site (13). We identified the gene responsible for kDNA loss as *CBRL*, which encodes a protein localized to the mitochondrion (Fig. 2B). A search of GenBank™ revealed CBRL orthologs in the kinetoplastid parasites *T. cruzi* (accession numbers 70878697 and 70882145 for a pair of proteins that differ in 12 of 388 amino acids, *E* values of *e*=146) and *L. major* (accession number 68124792, *E* value of *e*=110) but not in any other organism (the next nearest *E* value was *e*=18, for conventional *b*3Rs). Therefore, CBRLs appear to be specific to kinetoplastids. Nevertheless, the kinetoplastid CBRLs differ in structure. In addition to differences in sequence (the kinetoplastid CBRLs are 53–67% identical), the N-terminal domain contains 66 amino acids in *T. brucei* and *T. cruzi* but has only 12 in *L. major*.

Overexpression of CBRL had a novel effect on kDNA. During the course of 9 days of CBRL overexpression, DAPI staining of fixed cells revealed that kDNA networks gradually decreased in size and disappeared (Fig. 4B). Surprisingly this loss of kDNA networks was accompanied by only a small decrease in total minicircles as judged by Southern blot (Fig. 5B). These findings raised the possibility that the kDNA was decatenating or fragmenting with a majority of the minicircles being retained. This phenotype contrasts with that observed when other kDNA replication proteins were silenced by RNAi (4, 11, 12). In those cases total minicircles decreased in parallel with shrinking of the kDNA.
How can CBRL overexpression affect replication initiation? Previous reports have implicated redox in the regulation of DNA replication (32–34) and specifically in the case of UMSBP (28). Binding to the minicircle replication origin by UMSBP is dependent upon zinc knuckles, each containing three cysteines, making sulfhydryl oxidation an attractive method of regulation. Our finding that UMSBP is oligomerized, and presumably inactivated (28), upon overexpression of CBRL (Fig. 7) or tryparedoxin peroxidase (Fig. 8B) provides evidence that the redox state of UMSBP can be affected in vivo. With reduced levels of monomeric UMSBP, initiation of replication of covalently closed free minicircles would be inhibited, causing their accumulation (as shown in Fig. 6). The residual level of monomeric UMSBP could permit replication to occur at a reduced level, explaining the production of gapped minicircles. The unbalanced release and reattachment of minicircles from the network would result in its gradual shrinking (as shown in Fig. 4B).

The effect of CBRL overexpression on UMSBP could also explain the loss of maxicircles. Although relatively little is known about their replication, maxicircles contain two nearly perfect copies of the 12-bp universal minicircle sequence (UMS) separated by ~650 bp (35, 36). The UMS is the well characterized binding site for UMSBP. The two UMS-like sites in maxicircles differ in sequence from their minicircle counterpart by an adenine to guanine change in the terminal 3’ nucleotide, but this alteration does not affect UMSBP binding (37). Assuming that UMSBP is involved in maxicircle replication, its function probably differs from that in minicircles. In minicircles, leading strand DNA synthesis begins at the UMS, but in maxicircles replication initiates 2.5–3.0 kb downstream of the two UMS sequences (38). It is not known how, or whether, UMSBP binding could promote initiation of maxicircle replication. In any case, the rapid loss of maxicircles, which encode some respiratory chain proteins, could account for the rapid growth arrest. A similar loss of maxicircles combined with rapid growth arrest was observed when the mitochondrial RNA polymerase was silenced by RNAi (39).

How does overexpression of CBRL cause UMSBP oligomerization? Although we do not know the function of CBRL, its candidate NADH reductase; T, trypanthione; TPN, tryparedoxin; X<sup>red</sup>, an unknown substrate that is reduced by CBRL.

Network. It is likely that, during those previous RNAi experiments in which mRNA and protein levels decrease gradually, minicircles are lost by dilution as cells continue to divide for several days after kDNA synthesis is terminated. In contrast, minicircles are likely retained upon CBRL overexpression, which occurs quickly after induction, because of rapid cessation of cell growth (Fig. 1D).

Consistent with progressive network decatenation we found a 5-fold increase in free minicircles upon CBRL overexpression (Fig. 6B). A majority of free minicircles were covalently closed monomers, and there also was a significant increase in covalently closed dimers and other oligomeric minicircles (Fig. 6A). Other network fragments may have been too large to enter the gel. The covalently closed monomers could have been released from the network as part of the normal replication process. The oligomers could have been passively released from the progressively shrinking network. Although CBRL overexpression might somehow directly promote decatenation or fragmentation of the network, a much more likely explanation (based on current understanding of kDNA replication (6, 7)) is that the released covalently closed free minicircles accumulate because they fail to initiate replication.

How can CBRL overexpression affect replication initiation? Previous reports have implicated redox in the regulation of DNA replication (32–34) and specifically in the case of UMSBP (28). Binding to the minicircle replication origin by UMSBP is dependent upon zinc knuckles, each containing three cysteines, making sulfhydryl oxidation an attractive method of regulation. Our finding that UMSBP is oligomerized, and presumably inactivated (28), upon overexpression of CBRL (Fig. 7) or tryparedoxin peroxidase (Fig. 8B) provides evidence that the redox state of UMSBP can be affected in vivo. With reduced levels of monomeric UMSBP, initiation of replication of covalently closed free minicircles would be inhibited, causing their accumulation (as shown in Fig. 6). The residual level of monomeric UMSBP could permit replication to occur at a reduced level, explaining the production of gapped minicircles. The unbalanced release and reattachment of minicircles from the network would result in its gradual shrinking (as shown in Fig. 4B).

The effect of CBRL overexpression on UMSBP could also explain the loss of maxicircles. Although relatively little is known about their replication, maxicircles contain two nearly perfect copies of the 12-bp universal minicircle sequence (UMS) separated by ~650 bp (35, 36). The UMS is the well characterized binding site for UMSBP. The two UMS-like sites in maxicircles differ in sequence from their minicircle counterpart by an adenine to guanine change in the terminal 3’ nucleotide, but this alteration does not affect UMSBP binding (37). Assuming that UMSBP is involved in maxicircle replication, its function probably differs from that in minicircles. In minicircles, leading stand DNA synthesis begins at the UMS, but in maxicircles replication initiates 2.5–3.0 kb downstream of the two UMS sequences (38). It is not known how, or whether, UMSBP binding could promote initiation of maxicircle replication. In any case, the rapid loss of maxicircles, which encode some respiratory chain proteins, could account for the rapid growth arrest. A similar loss of maxicircles combined with rapid growth arrest was observed when the mitochondrial RNA polymerase was silenced by RNAi (39).
in T. cruzi increased resistance to exogenous peroxide; these authors did not report any effect on kDNA replication (40).

The mitochondrial tryparedoxin peroxidase may use a similar cascade involving NADPH, trypanothione, and tryparedoxin. We hypothesize that overexpression of mitochondrial tryparedoxin peroxidase or CBRL would deplete the mitochondrion of reduced tryparedoxin. If reduced tryparedoxin is required to maintain UMSBP in an active form (UMSBP), we assume that tryparedoxin peroxidase and CBRL have adequate substrate (ROOH and X^{-}, respectively, in Fig. 10) and that the re-reduction of tryparedoxin is slow. It is known that reduction of tryparedoxin by trypanothione is the rate-limiting step in the cascade (41). Furthermore, only a single trypanothione reductase has been identified in T. brucei, and it is localized in the cytosol (42). Therefore, re-reduction of tryparedoxin could be delayed by the requirement of transporting trypanothione to the cytosol. Alternatively, overexpressed CBRL or tryparedoxin peroxidase could simply bind and sequester tryparedoxin.

We provided direct evidence supporting the involvement of tryparedoxin by showing that the growth effects of CBRL overexpression can be rescued by simultaneously overexpressing tryparedoxin (Fig. 9A). Overexpressing tryparedoxin would prevent depletion of its reduced form, thereby maintaining UMSBP in its active form. Our data have provided further evidence that UMSBP, and therefore kDNA replication, might be regulated by redox. In vitro experiments had pointed toward tryparedoxin as a potential regulator (28), and our in vivo data support this idea. An alternate possibility is that UMSBP is not normally regulated by redox but is only maintained in an active form by reduced tryparedoxin. Therefore, depletion of reduced tryparedoxin causes UMSBP inactivation. Finally, this work indicates that overexpression of other genes might be a valuable genetic tool for studying trypanosome biology.

Acknowledgments—We thank Dr. Joseph Shlomai, Neta Millman-Shtepel, Dotan Sela, Dr. Barbara Sollner-Webb, Dr. Luise Krauth-Siegel, Dr. Juris Ozols, and Dr. Alan Fairlamb for helpful discussions or comments on the manuscript. We also thank Dr. Wade Gibson for the use of a phosphorimaging device.

REFERENCES

1. Sogin, M. L., and Silberman, J. D. (1998) Int. J. Parasitol. 28, 11–20
2. Stuart, K. D., Schnaufer, A., Ernst, N. L., and Panigrahi, A. K. (2005) Trends Biochem. Sci 30, 97–105
3. Drew, M. E., and Englund, P. T. (2001) J. Cell Biol. 153, 735–744
4. Wang, Z., and Englund, P. T. (2001) EMBO J. 20, 4674–4683
5. Melendy, T., Sheline, C., and Ray, D. S. (1988) Cell 55, 1083–1088
6. Liu, B., Liu, Y., Motyka, S. A., Agbo, E. E., and Englund, P. T. (2005) Trends Parasitol. 21, 363–369
7. Shlomai, J. (2004) Curr. Mol. Med. 4, 623–647
8. Wang, Z., Morris, J. C., Drew, M. E., and Englund, P. T. (2000) J. Biol. Chem. 275, 40174–40179
9. Morris, J. C., Wang, Z., Drew, M. E., and Englund, P. T. (2002) EMBO J. 21, 4429–4438
10. Drew, M. E., Morris, J. C., Wang, Z., Wells, L., Sanchez, M., Landefur, S. M., and Englund, P. T. (2003) J. Biol. Chem. 278, 46596–46600
11. Klingberg, M. M., Motyka, S. A., and Englund, P. T. (2002) Mol. Cell. 10, 175–186
12. Downey, N., Hines, J. C., Sinha, K. M., and Ray, D. S. (2005) Eur. J. Cell Biol. 4, 765–774
13. Motyka, S. A., Zhao, Z., Gull, K., and Englund, P. T. (2004) Mol. Biochem. Parasitol. 134, 163–167
14. Wirtz, E., and Clayton, C. (1995) Science 268, 1179–1183
15. Wirtz, E., Hoek, M., and Cross, G. A. (1998) Nucleic Acids Res. 26, 4632–4634
16. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
17. Brun, R., and Shonenberger, M. (1979) Acta Trop. 36, 289–292
18. van den Hoff, M. J., Moorman, A. F., and Lamers, W. H. (1992) Nucleic Acids Res. 20, 2902
19. Lamb, J. R., Fu, V., Wirtz, E., and Bangs, J. D. (2001) J. Biol. Chem. 276, 21512–21520
20. Church, G. M., and Gilbert W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1991–1995
21. Rusche, L. N., Cruz-Reyes, J., Piller, K. J., and Solnner-Webb, B. (1997) EMBO J. 16, 4069–4081
22. Shen, S., Arbin, G. K., Ullu, E., and Tschudi, C. (2001) Mol. Biochem. Parasitol. 113, 171–173
23. Bastin, P., Bagherzadeh, Z., Matthews, K. R., and Gull, K. (1996) Mol. Biochem. Parasitol. 77, 235–239
24. Colombo, S., Longhi, R., Alcaro, S., Ortuso, F., Sprocati, T., Flora, A., and Borgese, N. (2005) J. Cell Biol. 168, 735–745
25. Borgese, N., D’Arrigo, A., De Silvestris, M., and Pietrini, G. (1993) Subcell. Biochem. 21, 313–341
26. Tetaud, E., Giroud, C., Prescott, A. R., Parkin, D. W., Baltz, D., Bitoue, N., Baltz, T., and Fairlamb, A. H. (2001) Mol. Biochem. Parasitol. 116, 171–183
27. Klein, K. G., Olson, C. L., and Engman, D. M. (1995) Mol. Biochem. Parasitol. 70, 207–209
28. Onn, I., Milman-Shtepel, N., and Shlomai, J. (2004) Eur. J. Cell Biol. 3, 277–287
29. Wilkinson, S. R., Horn, D., Prathalingam, S. R., and Kelly, J. M. (2003) J. Biol. Chem. 278, 31640–31646
30. Tetaud, E., and Fairlamb, A. H. (1998) Mol. Biochem. Parasitol. 96, 111–123
31. Marchetti, A. A., Tschudi, C., Kwon, H., Wolin, S. L., and Ullu, E. (2000) J. Cell Sci. 113, 899–906
32. Wang, M., You, J. S., and Lee, S. H. (2001) Antioxid. Redox. Signal. 3, 657–669
33. You, J. S., Wang, M., and Lee, S. H. (2000) Biochemistry 39, 12953–12958
34. Konstantinov, Y., Tarasenko, V. I., and Rogozin, I. B. (2001) Dokl. Biochem. Biophys. 377, 82–84
35. Slood, P., de Haan, A., Eier, W., van Iersel, M., Boel, E., Van Steeg, H., and Benne, R. (1992) Mol. Biochem. Parasitol. 56, 289–299
36. Myler, P. J., Glick, D., Feagin, J. E., Morales, T. H., and Kelly, J. M. (1993) Nucleic Acids Res. 21, 687–694
37. Tzfati, Y., Abeiliovich, H., Kapeller, I., and Shlomai, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6891–6895
38. Carpenter, L. R., and Englund, P. T. (1995) Mol. Cell. 15, 6794–6803
39. Grams, J., Morris, J. C., Drew, M. E., Wang, Z., Englund, P. T., and Hajduk, S. L. (2002) J. Biol. Chem. 277, 16952–16959
40. Wilkinson, S. R., Temerton, N. J., Mondragon, A., and Kelly, J. M. (2000) J. Biol. Chem. 275, 8220–8225
41. Gommel, D. U., Nogoceke, E., Morr, M., Kiess, M., Kalisz, H. M., and Flohe, L. (1997) Eur. J. Biochem. 248, 913–918
42. Smith, K., Oppedoes, F. R., and Fairlamb, A. H. (1991) Mol. Biochem. Parasitol. 48, 109–112