Distinct cytoskeletal modulation and regulation of G1-S transition in the two life stages of *Trypanosoma brucei*

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Summary

Procyclic-form *Trypanosoma brucei* is arrested in G1 phase with extended and/or branched posterior morphology when expression of its cdc2-related kinases 1 and 2 (CRK1 and CRK2) is knocked down by RNA interference. Transmission electron microscopy indicated that the mitochondrion in the cell is also extended and branched and associated with cortical microtubules in each elongated/branched posterior end. This posterior extension is apparently driven by the growing microtubule corset, as it can be blocked by rhizoxin, an inhibitor of microtubule assembly. In the bloodstream form of *T. brucei*, however, a knockdown of CRK1 and CRK2 resulted only in an enrichment of cells in G1 phase without cessation of DNA synthesis or elongated/branched posterior ends. A triple knockdown of CRK1, CRK2 and CycE1/CYC2 in the bloodstream form resulted in 15% of the cells arrested in G1 phase, but no cells had an abnormal posterior morphology. The double and triple knockdown bloodstream-form cells were differentiated in vitro into the procyclic form, and the latter thus generated bore the typical morphology of a procyclic form without an extended/branched posterior end, albeit arrested in the G1 phase as the bloodstream-form precursor. There is thus a major distinction in the mechanisms regulating G1-S transition and posterior morphogenesis between the two life stages of *T. brucei*.

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Key words: CRK, Cell cycle, Cytoskeleton

Introduction

The African trypanosome, *Trypanosoma brucei*, is an infectious flagellated protozoan responsible for nagana in cattle and human sleeping sickness. It has a biphasic life cycle in the insect and mammalian host with profound changes in morphology, surface coat and metabolism during the transitions (Vickerman, 1985). The long slender bloodstream form and the insect (procyclic) form in the alimentary tract of the tsetse fly represent the two dividing forms of *T. brucei* that establish the infections.

*T. brucei* exhibits a specific range of cell morphologies defined by the internal cytoskeleton, which is characterized by a precisely arranged subpellicular corset of more than 100 microtubules that are crosslinked to each other and to the plasma membrane (Gull, 1999). These microtubules have their plus (+) ends all pointed toward the posterior end of the cell, consistent with a postulated unified direction of cortical microtubule extension (Robinson et al., 1995). Each cell possesses a single mitochondrion extending from the anterior to the posterior end of the cell. A mitochondrial DNA complex, termed the kinetoplast, has its own cell cycle coordinated with the nuclear cell cycle, with an S phase and the phase of kinetoplast segregation preceding the nuclear S phase and mitosis, respectively (Ploubidou et al., 1999; Woodward et al., 1990). A large body of evidence suggests that the kinetoplast cell cycle may not be totally inter-dependent with the nuclear cell cycle, and there exist different molecular mechanisms regulating the kinetoplast and nuclear cell cycles in *T. brucei* (Das et al., 1994; Ploubidou et al., 1999).

Regulatory pathways controlling the eukaryotic cell cycle have been studied in considerable detail in yeast and mammalian cells and shown to involve regulatory proteins such as cyclins and cyclin-dependent protein kinases (CDKs) (Mendenhall and Hodge, 1998). To date, five CDK homologues (designated cdc2-related kinases, CRK1, 2, 3, 4 and 6), four PHO80 homologues and three B-cyclin homologues have been identified in the *T. brucei* genome (Hammarton et al., 2003a). By RNA interference (RNAi) experiments, CRK3 and CycB2/CYC6 were found to control the G2-M checkpoint transition, whereas CRK1 and a Pho80 homologue CycE1/CYC2 were found to play important roles in the G1-S passage in both the procyclic and bloodstream forms of *T. brucei* (Hammarton et al., 2003b; Hammarton et al., 2004; Li and Wang, 2003; Tu and Wang, 2004). The procyclic form, when arrested in G1 phase by a double knockdown of CRK1 and CRK2, had an unusual morphology with an elongated and occasionally branched posterior end extended by newly synthesized microtubules (Hammarton et al., 2004; Tu and Wang, 2005). These findings suggested that posterior morphogenesis is coupled with G1-S passage in the
procyclic form, and that CRK2 may regulate the cell cycle-associated changes in the cytoskeleton.

To further understand the mechanism behind formation of the elongated/branched posterior end, the latter was examined by transmission electron microscopy and found to consist of the expected corset microtubule extension plus an elongated/branched mitochondrion. However, this morphological aberration was not observed in the bloodstream-form cells arrested in G1 phase, which, unlike the procyclic form, requires a triple knockdown of CRK1, CRK2, and CycE1/CYC2 to stop 15% of the cells from DNA synthesis completely. When the latter were differentiated in vitro under G1 arrest, the procyclic form thus produced had an apparently normal morphology of a procyclic form even though it was under G1 arrest as its bloodstream-form precursor. The data thus revealed a clear distinction in cell cycle regulation and cytoskeletal modulation between the two life stages of *T. brucei*.

**Materials and Methods**

**Cell lines and culture conditions**

The CRK1+CRK2-deficient procyclic-form *T. brucei* was cultivated at 26°C in Cunningham’s medium supplemented with 10% fetal bovine serum (Atlanta Biological) (Tu and Wang, 2005). G418 (15 μg/ml) and hygromycin B (50 μg/ml) were maintained in culture medium to preserve the T7 RNA polymerase and tetracycline repressor gene constructs within the cells. Phleomycin (2.5 μg/ml) was also added to preserve the pZJM vector after transfection.

The bloodstream form *T. brucei* strain 90-13 (Wirtz et al., 1999) was cultivated at 37°C in HM9 medium supplemented with 10% fetal bovine serum and 10% serum plus (JRH Biosciences) (Hirumi and Hirumi, 1989). G418 (2.5 μg/ml), hygromycin B (5 μg/ml) and phleomycin (2.5 μg/ml) were also added to stabilize the plasmids in the cell.

**Transmission electron microscopy**

Cells were fixed in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 hour at room temperature and post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2, for 45 minutes. Cells were spun onto poly-L-lysine-coated Aclar® tabs, dehydrated with ethanol, infiltrated with Epon and processed in a similar manner to the total RNA extract and incubated at 37°C for 2 minutes. Aclar® tabs were removed and cells of interest were identified using phase microscopy, marked using a diamond knife, cut out and remounted for sectioning. Ultrathin sections (50-65 nm) were collected and stained with uranyl acetate and lead citrate and imaged in a JEOL 1200 transmission electron microscope.

**Mitotracker staining**

CRK1+CRK2-deficient procyclic-form cells were induced by 1 μg/ml tetracycline for 5 days. Cells were harvested and suspended in fresh culture medium at a density of 1×10^6-1×10^7 cells/ml. Mitotracker® Green FM (Molecular Probe) was dissolved in dimethyl sulfoxide at 1 mM and added to a final concentration of 5 μM. The mixture was incubated for 20 minutes at 26°C, centrifuged, washed with fresh culture medium and incubated for another 20 minutes. Cell samples were collected, washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and fixed in 4% paraformaldehyde at 4°C for 15 minutes. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) in the presence of 1 μg/ml 4,6-diamino-2-phenylindole (DAPI) and examined with an Olympus phase-contrast and fluorescence microscope.

**Rhizinon treatment**

The CRK1+CRK2-deficient procyclic-form cells were treated with rhizinon at a final concentration of 1 nM and incubated for 5 days. Samples were collected and stained with the antibody YL1/2 (Chemicon, rat monoclonal antibodies against yeast tyrosinated α-tubulin, 1:400 dilution) or Mitotracker® Green FM as described previously (Tu and Wang, 2005). Slides were mounted in Vectashield in the presence of DAPI and examined with a fluorescence microscope.

**RNA interference (RNAi)**

Partial cDNA fragments (250-550 bp) of CRK1, CRK2, CRK4 and CRK6 (Trypanosome Genome Database accession numbers X64314, X74598, AJ413200 and AJ505556) were amplified by PCR using pairs of gene-specific primers (Tu and Wang, 2005), and ligated pairwise. Another partial fragment of CycE1/CYC2 (accession number AJ242519) nucleotides 26-420 was also amplified and ligated with CRK1 and CRK2 (sequences available upon request). All these combinations were cloned into the pZJM vector by replacing its α-tubulin fragment (Wang et al., 2000). The resulting RNAi constructs were linearized with NotI for integration into the *T. brucei* rDNA spacer region.

The fragment of a unique sequence from the coding region of each gene that has no significant sequence identity among the rest of the genome sequences in the Trypanosome Genome Database was ligated into three pairs (CRK1+CRK2, CRK1+CRK4, CRK1+CRK6) and a triplet (CycE1/CYC2+CRK1+CRK2) and subcloned into the RNAi vector pZJM (Tu and Wang, 2005; Wang et al., 2000). The newly generated sequences around the junctions of ligation in each combination were also examined in the genome database, and there was no significant sequence identity found among the rest of the genome sequences. It is thus unlikely that, by using these DNA constructs in RNAi experiments, expression of an unidentified gene could be inadvertently knocked down.

Transfection of the bloodstream-form *T. brucei* by electroporation was performed as previously described (Tu and Wang, 2004). The transfectants were selected with the addition of 2.5 μg/ml phleomycin and cloned on 0.6% agarose plates (Carruthers and Cross, 1992). The stable transfectants thus selected were grown in culture medium containing phleomycin. Transcription of the DNA insert was induced by adding 1 μg/ml tetracycline to the medium to switch on the T7 promoter. The effects of depleting multiple mRNAs on the growth of bloodstream-form trypanosome cells were monitored by a daily counting of the number of transfected cells using a hemocytometer.

**Semi-quantitative RT-PCR**

Total RNA was extracted from *T. brucei* cells using the TRIzol reagent (Amersharm Pharmacia). Before the PCR reaction, DNase I was added to the total RNA extract and incubated at 37°C for 30 minutes to remove the remaining DNA. A 100-500 ng total RNA sample was added to an RT-PCR using the one-step RT-PCR kit (Invitrogen) and ligated pair-wise. Another partial fragment of *TUB* (Trypanosome Genome Database accession numbers X64314, X74598, AJ413200 and AJ505556) were amplified by PCR using pairs of gene-specific primers (Tu and Wang, 2005), and ligated pairwise. Another partial fragment of CycE1/CYC2 (accession number AJ242519) nucleotides 26-420 was also amplified and ligated with CRK1 and CRK2 (sequences available upon request). All these combinations were cloned into the pZJM vector by replacing its α-tubulin fragment (Wang et al., 2000). The resulting RNAi constructs were linearized with NotI for integration into the *T. brucei* rDNA spacer region.

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Results

The elongated and/or branched posterior ends are filled with microtubules and an elongated and/or branched mitochondrion

Our previous work showed that in the procyclic form of T. brucei, a double knockdown of CRK1 and CRK2 arrested the cells in G1 phase. These G1 cells exhibited elongated and/or branched posterior ends, which, in an immunofluorescence assay, were found to be filled with newly synthesized microtubules (Tu and Wang, 2005). In the current study, we further examined the extended posterior ends by transmission electron microscopy and confirmed the presence of parallel microtubule arrays with a spacing between them equivalent to that observed in wild-type cells (Fig. 1B). However, each extension also contains another organelle that appears to be part of the mitochondrion in T. brucei (Fig. 1A). In order to verify this observation, we stained the cells with the mitochondrion specific dye, Mitotracker™, and examined them with a fluorescence microscope. The single mitochondrion in each cell is indeed elongated and branched into the elongated/branched posterior end all the way to the tip (Fig. 2).

Formation of the elongated and/or branched posterior end can be blocked by rhizoxin

There are two possible primary driving forces behind the cell cycle-dependent elongation of the posterior end. It could be by a continued growth of the microtubule corset followed by an extension of the mitochondrion associated with the microtubule arrays (Yaffe et al., 1996). Or less likely, it could be driven by the growing mitochondrial structure toward the posterior direction accompanied by microtubule elongation. To distinguish between these two possibilities, an anti-microtubule agent, rhizoxin (Ploubidou et al., 1999) was added at 1 mM to the cells at the beginning of RNAi induction for CRK1+CRK2 depletion. Five days later the cells had an apparently normal morphology with no sign of
elaganted/branched posterior ends (Fig. 3). They were further examined by immuno-fluorescence microscopy using YL1/2, an antibody specific for tyrosinated α-tubulin (Kilmartin et al., 1982; Wehland et al., 1983), which is present primarily in newly synthesized microtubules. It stains both the basal body and the newly assembled microtubules in the posterior end of T. brucei (Sherwin et al., 1987). The results showed no detectable staining of the posterior ends, which is similar to that observed in wild-type T. brucei cells (Fig. 3A). Mitotracker™ staining indicated that the mitochondrial structure also assumed a normal wild-type appearance with no sign of extended growth at the posterior end after rhizoxin treatment (Fig. 3B). BrdU incorporation analysis indicated that the rhizoxin treatment of CRK1+CRK2-depleted cells did not resume the nuclear DNA synthesis (supplementary material Fig. S1). RT-PCR showed that the expression of CRK1 and CRK2 remained both knocked down in the presence of rhizoxin (supplementary material Fig. S2). Thus, these data suggest that the elongated/branched posterior end of a CRK1+CRK2-deficient procyclic-form G1 cell is primarily attributed to an uncontrolled extension of

**Fig. 2.** Mitochondrial staining of CRK1+CRK2-deficient procyclic-form cells. CRK1+CRK2-deficient procyclic form and control cells were stained by Mitotracker™ Green FM (Mitotracker) and DAPI. The single mitochondrion extends from one end to the other of the wild-type cell. An elongated and/or branched mitochondrion is seen filling the elongated/branched posterior end of the CRK1+CRK2-deficient cell.

**Fig. 3.** Rhizoxin shortens the posterior ends of CRK1+CRK2-deficient procyclic-form cells. Rhizoxin (1 nM) was added to the CRK1+CRK2-deficient procyclic-form cells at the same time as tetracycline. The cells were cultured for 5 days, collected and stained with DAPI, YL1/2 antibody (A) and Mitotracker™ Green FM (B).
the corset microtubules toward the posterior direction. The mitochondrial structure is probably simply expanded to fill the available space.

In the bloodstream form of *T. brucei* a knockdown of CRK1 and CRK2 only enriched cells in the G1 phase without inhibiting nuclear DNA synthesis. To verify if the unusual coupling between posterior morphogenesis and G1-S transition observed in the procyclic form also applies to the bloodstream form of *T. brucei*, we tried first to use the RNAi technique to arrest the latter effectively in the G1 phase. Our previous effort showed that a knockdown of CRK1 in the bloodstream form reduced cell growth to 17% of the control with an increase of G1-phase cells from 45% to 60% and a corresponding decrease of S-phase cells from 43% to 28% without appreciable change in the G2-M population (Tu and Wang, 2004). Those data suggested that a slowing down of G1-S passage resulted from CRK1 depletion and an important role played by CRK1 in regulating this passage in the bloodstream form. For a more complete cell arrest in the G1 phase, we tried RNAi pair-wise knockdowns including CRK1 and another CRK2, 4 or 6 in the bloodstream form. CRK3 was not included, because it is known to have a specific function in regulating only the G2-M transition (Tu and Wang, 2004).

Experimental results indicated that a knockdown of CRK1+CRK2 in the bloodstream form led to more significant growth arrest than if CRK1 was knocked down alone (Fig. 4A). The number of double knockdown cells reached less than 6% of that in the uninduced control after 4 days of RNAi. The generation time was calculated to increase from 7.5 hours in the control to 11 hours. This enhanced growth arrest by the combined knockdowns may suggest that CRK1 and CRK2 play a redundant role in regulating the G1-S passage. For CRK1+CRK4 and CRK1+CRK6 double knockdowns, a growth inhibition similar to that of CRK1 knockdown alone was observed (data not shown), which agrees with the finding from our previous studies that CRK4 and CRK6 do not play any apparent role in cell cycle regulation in *T. brucei* (Tu and Wang, 2004).

FACS analysis of the CRK1+CRK2 knockdown cells by their DNA contents indicated that after RNAi for 3 days, the G1 cells were increased from 48% to 74% of the population, S-phase cells decreased from 38% to 19% whereas the G2/M-phase cells decreased from 14% to 7% (Fig. 4B). The data represent a more extensive arrest of cells in the G1 phase when compared with that from knockdown of CRK1 alone (Tu and Wang, 2004).

Propidium iodide-stained cells were examined for those containing one nucleus-one kinetoplast (1N1K), one nucleus-two kinetoplasts (1N2K), two nuclei-two kinetoplasts (2N2K), no nucleus-one kinetoplast (0N1K, the zoid) and multiple nuclei-multiple kinetoplasts (XNXK). The CRK1+CRK2 knockdown resulted in a 10% increase in 1N1K cells from 78% to 88% with a corresponding 4% decrease in 1N2K and 6% decrease in 2N2K cells without detectable emergence of either zoids or XNXK cells (Fig. 5A).

The 74% of G1 cells estimated by FACScan and the 88% of 1N1K cells by fluorescence microscopy 4 days following CRK1+CRK2 knockdown suggested a majority of cells arrested in the G1 phase. However, data from BrdU incorporation from day 3 to day 5 after RNAi initiation indicated that essentially all the 1N1K cells are still capable of synthesizing DNA (Fig. 5B). The G1 cells are thus not truly arrested in this phase but, rather, progressing through the G1-S checkpoint at a slower rate. This is quite different from that observed in the procyclic form of *T. brucei*, in which a CRK1+CRK2 knockdown led to 50% of the population arrested in G1 phase incapable of DNA synthesis (Tu and Wang, 2005). These discrepant data suggest that there are

![Fig. 4. Effects of double knockdowns of CRK1 and CRK2 on the growth and cell cycle progression in bloodstream-form cells. (A) Cloned bloodstream trypanosome cells harboring the CRK1+CRK2 RNAi plasmid construct were incubated in culture medium at 37°C without (-Tet) or with 1.0 μg/ml tetracycline (+Tet). Cell growth was monitored daily and cell numbers plotted on a logarithmic scale. The inset shows the semi-quantitative RT-PCR assessment of intracellular mRNA levels after a 3-day RNAi induction. α-Tubulin mRNA (TUB) was included as a sampling control. (B) Samples of the CRK1+CRK2 knockdown cells over 4 days were stained with propidium iodide and subjected to FACScan analysis for DNA content. The histograms from the FACScan are presented on the left and the percentages of cells in G1, S and G2/M phases determined by the ModFitLT software are plotted on the right.](image-url)
Fig. 5. Effect of knocking down CRK1 and CRK2 on the numbers of nuclei and kinetoplasts and BrdU incorporation in bloodstream-form cells. (A) 3 days after RNAi induction, cells were stained with propidium iodide and examined with a fluorescence microscope for tabulation of cells with different numbers of nuclei (N) and kinetoplasts (K). Data are presented as the mean percentage (±s.e.) of total cells counted (>200) from three independent experiments. (B) BrdU incorporation analysis. The double knockdown cells were labeled with BrdU 3-5 days after the initiation of RNAi and examined by immunofluorescence assay.

Fig. 6. Effects from double knockdowns of CRK1 and CRK2 on the morphology of bloodstream-form cells. (A) Cells 3 days after RNAi induction were stained with propidium iodide and examined with a fluorescence microscope. Upper panel, 1N1K, 1N2K and 2N2K control cells without RNAi induction. Lower panel, CRK1+CRK2-depleted 1N1K, 1N2K and 2N2K cells. (B) A CRK1+CRK2-depleted cell stained with YL1/2 antibody, showing the absence of newly synthesized microtubule from the posterior end.
distinctive molecular mechanisms regulating cell cycle progression in the two developmental stages of *T. brucei*.

The CRK1 and CRK2 double knockdown does not lead to an elongated posterior end in the bloodstream form Morphology of the CRK1+CRK2-deficient bloodstream-form cells was found to be normal and indistinguishable from the uninduced control cells (Fig. 6A). Cells stained with the YLI1/2 antibody against newly synthesized microtubules were further examined by immunofluorescence assay and only the basal bodies were found to be stained (Fig. 6B). There was no heavily stained posterior end, which was in agreement with that observed in the control cells.

A CycE1/CYC2, CRK1 and CRK2 triple knockdown fails to show any morphological change in the bloodstream form As a previous knockdown of the expression of CycE1/CYC2 from the procyclic form of *T. brucei* resulted in G1 arrest accompanied with an elongated posterior end (Li and Wang, 2003), we tested the same knockdown in the bloodstream form and noticed only a partial enrichment of G1 cells without any indication of inhibited DNA synthesis or morphological change (Z. Li and C.C.W., unpublished). In a further attempt to arrest the bloodstream-form cells in the G1 phase, we performed a triple knockdown of CycE1/CYC2, CRK1 and CRK2 by RNAi. The number of triple knockdown cells was only 2% of that in the uninduced control after 4 days of RNAi induction. The generation time of these triple knockdown cells was increased from 7.5 hours in the control to 13 hours (Fig. 7A), which is longer than the 11-hour generation time for the CRK1+CRK2 double knockdown cells (Fig. 4A). The number of G1 cells increased from 45% to 75%, S-phase cells decreased from 38% to 16% whereas the number of G2-M-phase cells reduced from 14% to 6% (Fig. 7B). The data are consistent with those from the karyotype distribution study which showed a 74% to 90% increase of 1N1K cells accompanied by a corresponding 14% to 8% decrease in 1N2K and a reduction from 12% to 2% in 2N2K cells (Fig. 7C). There was no zoids or XNXK cells detectable in the triple knockdown population.

This further enrichment of bloodstream-form cells in the G1 phase through a triple knockdown was verified by data from BrdU incorporation during day 3 to day 5 of the RNAi induction (Fig. 7D). Approximately 15% of the cells (based on a total count of 600 cells from three independent experiments) were incapable of incorporating a detectable amount of BrdU, indicating that these cells were probably truly arrested in the G1 phase. A microscopic examination of these cells indicated that they had a normal morphology when compared with the control cells (Fig. 7D). The YLI1/2 antibody stained only the basal bodies in these cells (Fig. 7E). There is thus little doubt that, unlike the procyclic-form cells trapped in G1 phase, the bloodstream form arrested in G1 phase has no detectable morphological change and no elongated/branched posterior end.

**Discussion**

The elongated/branched posterior end in CRK1+CRK2-depleted procyclic form *T. brucei* is filled with microtubule corset and extended mitochondrial structure In the present study we examined the ultrastructure of the posterior end of CRK1+CRK2-depleted procyclic-form *T. brucei* to verify that the extended/branched posterior end is filled with the microtubule corset (Tu and Wang, 2005). The analysis also demonstrated the presence of mitochondrial structure in the extended and branched posterior ends of the G1-arrested cells. The single mitochondrion in each *T. brucei* cell is extended all the way from the anterior end to the posterior end (Vassella et al., 2003).
Fig. 7. See next page for legend.
Distinct cytoskeletal modulation between two life cycle forms of *T. brucei*

The cytoskeletal modulation in procyclic-form *T. brucei* apparently does not apply to the bloodstream form of *T. brucei*. When CRK1 and CRK2 were depleted simultaneously in the latter, the cells have an apparently normal morphology without an extended/branched posterior end. As our previous knockdown of CycE1/CYC2 from the procyclic form resulted in cells with elongated posterior ends (Li and Wang, 2003), we performed a triple knockdown of CycE1/CYC2+CRK1+CRK2 in the bloodstream form with 90% of the cells in the G1 phase, but it still resulted in cells with normal morphology.

One significant morphological difference between the two forms of *T. brucei* lies in the position of the kinetoplast. In the procyclic form, the kinetoplast is located midway between the nucleus and the posterior end, whereas in the bloodstream form, the kinetoplast is located at the extreme posterior end of the cell. Differentiation of the bloodstream form into the procyclic form requires the repositioning of the kinetoplast to a location midway between the nucleus and cell posterior (Matthews et al., 1995). This repositioning may require cytoskeletal changes involving the extension of microtubule corset toward the posterior end of the cell (Hendriks et al., 2001). The requirement for this cytoskeletal modulation reflects the need for one of the two nuclei to migrate between the two segregated kinetoplasts prior to cytokinesis in the procyclic form (Sherwin and Gull, 1989; Woodward and Gull, 1990). But such a migration of the nucleus is apparently not required prior to cytokinesis in the bloodstream form (Hammarton et al., 2003a).

Thus, a posterior extension may become an essential step in the procyclic form during the G1 phase to reposition the kinetoplast, which is apparently ended by the action of CRK2 during the G1-S transition (Tu and Wang, 2005). But a similar event does not occur in the bloodstream form. Thus a knockdown of CRK2 expression in the latter does not result in an elongated/branched posterior end.

However, this explanation is challenged when the CRK1+CRK2 double knockdown and CRK1+CRK2+CycE1/CYC2 triple knockdown bloodstream form cells differentiated into the procyclic form without showing any elongated/branched posterior ends or any other abnormal morphology. A careful examination of the positions of kinetoplasts in these newly formed procyclic-form cells indicated that they have moved midway between the nuclei and the posterior ends as seen in typical procyclic form cells (Fig. 8B and supplementary material Fig. S3). This migration of kinetoplast together with the loss of VSG221 and emergence of procyclin indicate normal progression of differentiation while the cells are under G1 arrest. But it is not necessarily an indication of completion of the differentiation process. Although not coupled to cell cycle progression, this process is believed to represent a change in the profile of gene expression when the environmental temperature drops from 37°C to 26°C (Diehl et al., 2002). The change is attributed primarily to a post-transcriptional alteration of mRNA stability involving specific regions in the 3’-untranslated regions of the mRNA and the corresponding binding proteins (Blattner and Clayton, 1995), which may occur throughout the G1, S, G2 as well as M phases in the cell cycle. The arrest of cells in G1 via the induction of VSG221, a typical procyclic form cell surface antigen, and the concomitant appearance of procyclin indicate normal progression of differentiation while the cells are under G1 arrest.
Fig. 8. See previous page for legend.
phase may prevent the completion of a total transformation from bloodstream to procyclic form, because the necessary post-transcriptional events that occur only in the cell cycle phases other than G1 are not accomplished. Thus, the procyclic-form cells derived from differentiation under G1 arrest may have acquired only part of the procyclic characteristics, which may explain their similarities to the bloodstream form in terms of nuclear DNA synthesis and a lack of morphological change.

Overexpression of a differentiation-associated CCCH zinc finger protein TbZFP2 in the bloodstream form leads to an elongated posterior morphology in the differentiated procyclic-form cells (Hendriks et al., 2001). It is likely that TbZFP2 is involved in the posterior extension of the microtubule corset, which probably repositions the kinetoplast during differentiation. A knockdown of TbZFP2 expression would thus predict a failure in kinetoplast repositioning during differentiation resulting in a ‘procyclic form’ that retains some of the bloodstream characteristics.

Different G1-S checkpoint regulations between procyclic and bloodstream forms of T. brucei

Another distinction between the two forms of T. brucei is reflected in the apparently more complex mechanisms of regulating G1-S passage in the bloodstream form. Although a CRK1+CRK2 knockdown resulted in an 80% population in G1 phase and 50% of the population incapable of nuclear DNA synthesis in the procyclic form (Tu and Wang, 2005), the same knockdown led to a 74% population in G1 phase with essentially all the cells still capable of nuclear DNA synthesis in the bloodstream form. Even in the CRK1+CRK2+CyE1/CYC2 triple knockdown with 90% of the bloodstream-form cells retained in G1 phase, only 15% of the cells were incapable of incorporating BrdU. This remarkable ability of continued nuclear DNA synthesis indicates continued passage from G1 into S phase despite depletion of the cyclin and CRKs known to control G1-S transition in T. brucei (Li and Wang, 2003; Tu and Wang, 2004; Tu and Wang, 2005). More proteins may be involved in controlling G1-S transition in the bloodstream form that remain to be identified.

In summary, we observed that the elongated and/or branched posterior ends in CRK1+CRK2-deficient procyclic-form cells are filled with microtubules accompanied with mitochondrion structure. This morphological aberration is, however, not observed in the bloodstream form or the procyclic form derived from differentiation of the bloodstream form depleted of CRK1+CRK2 or CRK1+CRK2+CyE1/CYC2. The regulation of G1-S transition has thus an apparently much more complex mechanism in the bloodstream forms.

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