The Involvement of Two cdc2-related Kinases (CRKs) in Trypanosoma brucei Cell Cycle Regulation and the Distinctive Stage-specific Phenotypes Caused by CRK3 Depletion*

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Cyclin-dependent protein kinases are among the key regulators of eukaryotic cell cycle progression. Potential functions of the five cdc2-related kinases (CRK) in Trypanosoma brucei were analyzed using the RNA interference (RNAi) technique. In both the procyclic and bloodstream forms of T. brucei, CRK1 is apparently involved in controlling the G1/S transition, whereas CRK3 plays an important role in catalyzing cells across the G2/M junction. A knockdown of CRK1 caused accumulation of cell cycle control among the eukaryotes. It suggests leakiness in the mechanism of trypanosome cell division, a phenomenon not yet observed among other eukaryotes. Procyclic and bloodstream forms. The procyclic form has a majority of the cells containing a single enlarged nucleus plus one kinetoplast. There is also an enhanced population of anucleated cells, each containing a single kinetoplast known as the zoids (0N1K). The CRK3-depleted bloodstream form has an increased number of one nucleus-two kinetoplast cells (1N2K) and a small population containing aggregated multiple nuclei and multiple kinetoplasts. Apparently, these two forms have different mechanisms in cell cycle regulation. Although the procyclic form can be driven into cytokinesis and cell division by kinetoplast segregation without a completed mitosis, the bloodstream form cannot enter cytokinesis under the same condition. Instead, it keeps going through another G1 phase and enters a new S phase resulting in an aggregate of multiple nuclei and multiple kinetoplasts in an undivided cell. The different leakiness in cell cycle regulation between two stage-specific forms of an organism provides an interesting and useful model for further understanding the evolution of cell cycle control among the eukaryotes.

The progression of the eukaryotic cell cycle is dictated by an ordered series of checkpoints, each regulated by multiple, but well conserved, regulatory proteins. They exert a tight control over DNA replication, mitosis, cytogenesis, and cell division resulting in maintenance of genomic stability. Through the well controlled periodic synthesis and destruction of various cyclins, the cyclin-dependent protein kinase (CDK)1 activity goes through a corresponding sequential fluctuation, which provides the primary means of cell cycle control (1). In Saccharomyces cerevisiae, there are three G1 cyclins (CLN1–CLN3) and six B-type mitotic cyclins (CLB1–CLB6) that interact with a single CDK, CDC28, in a sequential and overlapping manner to activate the latter for regulating the yeast cell cycle (2–4). In mammalian cells, there have been at least 9 CDK and 16 cyclin homologues identified. Although not all of them are involved in directing the cell cycle, the overlapping regulatory functions already identified among many of these proteins in cell cycle progression suggest a most complex controlling system (1).

Trypanosoma brucei is a parasitic protozoan and the causative agent of sleeping sickness in Africa. It is also generally regarded as a deeply branched and relatively primitive eukaryote further removed from mammals than yeast. The trypanosomes have a complex life cycle of distinctive stage-specific forms that alternate between an insect vector, the tsetse, and a mammalian host. The bloodstream form inhabiting the mammalian blood and the procyclic form in the midgut of tsetse are known to differ significantly in many biochemical and metabolic aspects due to their distinctive living environments (5). The trypanosome cell cycle in both bloodstream and procyclic forms has the usual sequential G1, S, G2, and M phases (6). But it differs also from yeast and mammalian cells by the presence of a single mitochondrion in each cell, which divides synchronously with the nucleus (7). The mitochondrial DNA complex, the kinetoplast, has its own cell cycle with an S phase (Sp) and the phase of kinetoplast segregation preceding the nuclear S phase (S(np)) and mitosis, respectively (6, 7). The kinetoplast is closely associated with the flagellum basal body, and its segregation depends on the microtubule-mediated separation of replicated basal bodies, which represent the initial step of trypanosome cell division (8). Kinetoplast segregation is presumably well coordinated with mitosis initiating and leading the cell through cytokinesis and eventual division (8). Recent experimental evidence suggested, however, that these two events may not be inter-dependent of each other in procyclic form trypanosome, because okadaic acid treatment prevented the kinetoplast from replicating but resulted in multinucleated cells (9). Treatment with the nuclear DNA synthesis inhibitor aphidicolin or the antimicrotubule agent rhizoxin resulted in blocked mitosis without inhibiting kinetoplast segregation, which led to cytokinesis and cell division, generating anucleated daughter cells designated as the zoids (7, 10). Thus the kinetoplast cycle alone can apparently drive the procyclic form into cell division, a phenomenon not yet observed among other eukaryotes. It suggests leakiness in the mechanism of trypanosome cell cycle regulation.

1 The abbreviations used are: CDK, cyclin-dependent kinase; CRK, cdc2-related kinase; RNAi, RNA interference; BrdUrd, 5-bromo-2-deoxyuridine; FACS, fluorescence activated cell sorting; DAPI, 4,6-diamino-2-phenylindole; GPI, glycosylphosphatidylinositol; PI, propidium iodide; RT, reverse transcriptase.

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There are four PHO80 cycin homologues, three B-type mitotic cycin homologues, but no G_{1} cycin homologue in the genome of T. brucei (11). In the procyclic form of trypanosome, one of the PHO80 homologues CycE1/CYC2 plays an essential role in controlling the G_{1}/S passage, whereas the transition through G_{2}/M is regulated by a single B-type mitotic cycin CycB2/CYC6 (11). By using the technique of RNA interference (RNAi) in T. brucei, depletion of CycE1/CYC2 resulted in an enrichment of slender-shaped anucleated zoids, whereas a reduced C CycB2/CYC6 level led to an enhanced population of stumpy-shaped zoids (11). These results reiterated that, in the procyclic form, the kinetoplast cycle alone can drive the cells blocked in S_{0} or mitosis through cytokinesis to cell division. But in a separate study, a knockdown of CycB2/CYC6 from the bloodstream form generated cells of one nucleus and multiple kinetoplasts without any anucleated zoids (12). Thus, kinetoplast replication and segregation apparently continue in the absence of mitosis in the bloodstream form too, but they are incapable of bringing about cell divisions. These distinctive regulatory mechanisms of cell division demonstrated in two different developmental stages of the same living organism represent yet another unusual biological phenomenon in T. brucei not reported previously on other eukaryotes.

The potential CDKs involved in regulating cell cycle progression in T. brucei have not yet been identified. Several CDK homologues in the T. brucei genome have been cloned from the previous studies and designated the cdc2-related kinases (CRKs) (13, 14). Among them, CRK1-4 and 6 have several features in common with the CDKs from yeasts and mammals, including an overall 40–50% amino acid sequence identity, a recognizable "PSTAIR" cyclin-binding box, and a "DEI" box and the key threonine and tyrosine residues known as the important phosphorylation sites (13). There are also sequence features in these CRKs that distinguish them from yeast and mammalian CDKs, including an N-terminal extension in CRK2 and CRK3 and two additional domains within the catalytic site of CRK4 (13). Although sequence data alone cannot distinguish which CRK is involved in regulating the cell cycle, CRK3 was found to complex with CycE1/CYC2 (15) as well as CycB2/CYC6 (12) in yeast two-hybrid tests, thus suggesting its involvement in cell cycle control.

In our present study, we used the RNAi technique to knockdown the respective expression of CRK1–4 and 6 in the procyclic as well as the bloodstream forms of T. brucei. Data thus assembled indicated that CRK1 plays a role in regulating the G_{1}/S passage, whereas CRK3 is involved in G_{2}/M transition in both forms of trypanosome. The cell cycle blockade resulted from CRK3 depletion led to an accumulation of zoids in the procyclic form, but only an enrichment of cells with multinucleated aggregates and multiple kinetoplasts was observed in the bloodstream form. This outcome not only reiterates the previous observations from cyclin knockdown studies (11, 12) but also reveals an important role of CRK3 in completing the mitosis required for cell division in the bloodstream form but apparently not in the procyclic form. There is thus a difference in the mechanisms of regulating cell division between the two developmental forms of trypanosome.

**MATERIALS AND METHODS**

**Cell Culture**—The procyclic form T. brucei strain 29-13 (16) was cultivated at 26 °C in the Cunningham’s medium supplemented with 10% fetal bovine serum (Atlanta Biological). G418 (15 μg/ml) and hygromycin B (50 μg/ml) were maintained in the culture medium to preserve the TT RNA polymerase and tetracycline repressor gene constructs within the cells.

The bloodstream form T. brucei strain 90-13 (16) was cultivated at 37 °C in the HMI9 medium supplemented with 10% fetal bovine serum and 10% serum plus (JRHI Biosciences) (17). G418 (2.5 μg/ml) and hygromycin B (5 μg/ml) were also added to the culture medium to maintain the TT RNA polymerase and tetracycline repressor gene constructs in the bloodstream form.

**RNA Interference**—A partial cDNA fragment (250–550 bp in length) of each of the five T. brucei CRK genes was used for RNAi. (The accession numbers of CRK1, CRK2, CRK3, CRK4, and CRK6 genes are X64314, X74598, X74617, AJ413200, and AJ505556, respectively.) The sequences used for RNAi were nucleotide numbers 12–483 for CRK1, 468–906 for CRK2, 427–797 for CRK3, 91–650 for CRK4, and 91–520 for CRK6. Each DNA fragment was amplified by PCR using a pair of gene-specific primers with respective XhoI and HindIII linkers and subcloned into the pZJM vector by replacing the α-tubulin fragment in it (18). The resulting RNAi construct was linearized with NotI for integration into the rDNA spacer region in T. brucei chromosome. Transfection of the procyclic form T. brucei with the linearized RNAi construct by electroporation was performed essentially according to the procedures described previously (19, 20). The transfectants were selected under 2.5 μg/ml phleomycin, and single transfected cells were cloned by limiting dilutions. To induce RNAi, the cloned stable transfectants were cultured in the presence of 1.0 μg/ml tetracycline.

Transfection of the bloodstream form T. brucei by electroporation was performed as described previously with some modifications (21). Briefly, a sample of 1 × 10^{7} log phase cells were harvested, washed once with cytometry buffer (22), and suspended in 0.5 ml of the same buffer containing 100 μg of the linearized pZJM DNA construct described above. Electroporation was carried out in a 4-mm cuvette using the Gene Pulser (Bio-Rad) with parameters set as follows: volt age, 400-ohm resistance, and 25-microfarad capacitance. The cells were transferred to a 24-well plate in HMI 9 medium immediately after electroporation and incubated at 37 °C for 24 h. The transfectants were then selected with the addition of 2.5 μg/ml phleomycin, and single cells were cloned by limiting dilutions. For induction of RNAi, the cloned stable transfectants were cultured in the presence of 1.0 μg/ml tetracycline. Cell numbers were counted under microscope at different time intervals using a hemocytometer.

**Semi-quantitative RT-PCR**—Total RNA was extracted from T. brucei cells using the TRizol reagent (Amersham Biosciences). First-strand cDNAs were generated from DNase I-treated RNA samples using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega). PCR was then performed using the first-strand cDNA and a pair of gene-specific primers that differs from the primer pair used in generating the original RNAi construct (sequences available upon request).

**Fluorescence-activated Cell Sorting (FACS) Analysis**—Cell samples for FACS analysis were prepared as described previously (22) with minor modifications. Briefly, time samples of the transfected T. brucei cells (2 × 10^{7} cells) were collected before and during tetracycline induction, centrifuged at 2500 × g and 4 °C for 10 min, and washed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 8 mM KCl, 10 mM Na_{2}HPO_{4}, 2 mM KH_{2}PO_{4}, pH 7.4). The cell pellets were gently suspended in 100 μl of PBS containing 1.5% in 107 log phased cells were harvested, washed with PBS twice and resuspended in PBS. The same PI-stained cell samples were also examined under 2.5 μg/ml tetra cycline. Cell numbers were counted under microscope at different time intervals using a hemocytometer.

**BrdUrd Labeling of Cells**—BrdUrd was added to the suspension to the final concentrations of 10 and 20 μg/ml, respectively, and incubated for 30 min at room temperature before the FACS analysis. The DNA content of PI-stained cells was analyzed with a FACSCan analytical flow cytometer using the Cellquest software (BD Biosciences). Percentage of cells in each phase of the cell cycle, G_{1}, S, and G_{2}/M, was determined by the ModFitLT version 3.1 software (BD Biosciences). Percentages of cells in each phase of the cell cycle, G_{1}, S, and G_{2}/M, was determined by the ModFitLT version 3.1 software (BD Biosciences). Briefly, BrdUrd for T. brucei was included in the manufacturer’s protocol (BD Biosciences). Briefly, the DNA in fixed cells was first denatured in 2 × HCl at room temperature for 20 min, washed, and neutralized in 0.1 M sodium borate. Mouse anti-BrdUrd polyclonal antibody (Sigma) diluted 1:100 in PBS. 0.5% bovine serum albumin was then added and incubated at room temperature for 1 h, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Sigma) diluted 1:70 in PBS, 0.5% bovine serum albumin for another hour. Slides were mounted in
RESULTS

RNA Interference of the Expression of Individual CRK Genes in T. brucei—We employed the RNAi technique to selectively knockdown expression of each of the five individual CRK genes both in the procyclic and bloodstream forms of T. brucei to explore the potential role each may play during the cell cycle. A 250–500-bp DNA 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 Trypanosome Genome Data base was amplified by PCR and subcloned into the RNAi vector pZJM (18). The resulting construct was linearized and introduced by electroporation into either the procyclic form cells of T. brucei strain 29-13 or the bloodstream form cells of T. brucei strain 90-13 expressing both tetracycline repressor and T7 RNA polymerase. The stable transfectants in both forms were selected under phleomycin and cloned by limiting dilutions. Transcription of the DNA insert into double-stranded (ds) RNA was induced by adding tetracycline to the culture medium to switch on the T7 promoters, and the double-stranded RNA thus synthesized is known to lead to specific degradations of its corresponding mRNA in T. brucei (23–26).

The effect of RNAi on individual CRK gene expression was first examined by semi-quantitative RT-PCR analysis. The results (shown in the insets of Figs. 1A and 3) indicate that, after initiating RNAi for 3 days, the levels of mRNAs encoding individual CRKs were decreased significantly in both procyclic and bloodstream forms. This knockdown of gene expression was highly specific. Only a single mRNA species designated for the knockdown was diminished, whereas levels of the other CRK mRNAs remained unchanged in each case. Two such examples for the procyclic form of T. brucei are presented in Fig. 1B, in which RT-PCR analysis shows that a CRK1 knockdown results only in a decreased level of CRK1 mRNA, whereas a CRK3 knockdown leads to the disappearance of only CRK3 transcript. The effects of individual CRK mRNA depletion on growth of trypanosome cells were then monitored by a daily counting of the number of transfected cells with or without tetracycline induction (Figs. 1A and 3). Due to their different generation times, the slower growing procyclic form was cultivated for 9 days, whereas the bloodstream form was grown for 4 days in these experiments.

Effects of Individual CRK Depletions on the Cell Cycle Progression of Procytic Form T. brucei—The results in Fig. 1 indicate that the growth rates of CRK1-, CRK3-, and CRK4-deficient cells were reduced to 19, 9, and 36% of the RNAi un-induced controls, respectively, in the procyclic form. Growth of cells depleted of either CRK2 or CRK6 appeared apparently the same rate as those cells growing without an induced RNAi. FACS analysis of the cell populations by their DNA contents indicated that 9 days after knocking down the expression of CRK1, there was an increase of about 10% cells in the G1 phase accompanied with a corresponding 10% decrease from the S phase, whereas the percentage of G2/M phase cells remained relatively unchanged (Fig. 2). The data can be taken as an indication that CRK1 plays a role in facilitating the cells across the G1/S checkpoint. Among the CRK3-depleted cells, those originally in the G1 phase were decreased from 45 to 15% of the total population, whereas the G2/M phase cells were increased from 20 to 50%, and the S phase cell population was not significantly changed (Fig. 2). CRK3 thus may perform an important function in catalyzing the procyclic form cells through G2/M transition. When CRK4 was deficient, there was relatively little change in the percentages of cells in different phases of the cell cycle as compared with the control (Fig. 2). It could be that CRK4 is not involved in cell cycle regulation at all, but the 64% inhibition of growth rate caused by its knockdown (Fig. 1) may also suggest that CRK4 is involved in controlling passage of cells through both G1/S and G2/M junctions. There were some minor fluctuations of cell population in the three cell cycle phases during the 9-day incubation when either CRK2 or CRK6 was deficient (Fig. 2). Because the rates of growth were unaffected by their removal (Fig. 1A), these two CRKs are most likely not involved or playing overlapping functions with other proteins in cell cycle regulation in the procyclic form of T. brucei.

Effects of Individual CRK Depletions on the Cell Cycle Progression of Bloodstream Form T. brucei—In the bloodstream form, a knockdown of CRK1, CRK3, and CRK4 expression by RNAi resulted in reduced rates of growth of 17, 31, and 68% of the un-induced control, respectively, whereas a loss of CRK2 or CRK6 exerted no appreciable effect on growth rate (Fig. 3). When CRK1 was depleted from the cells, there was an increase of G1 phase cells (from 45 to 60%), accompanied by a corresponding decrease of S phase cells from 43 to 28% without appreciable change in the G2/M population over a period of 3 days. The data suggest that the transition from G1 to S phase is slowed down, and CRK1 may thus perform an important role in the G1/S transition in the bloodstream form as in the procyclic form (Fig. 2). Among the CRK3-deficient cells, those in the G1 phase were reduced from 50 to 30% of the total population, whereas the G2/M phase cells were enriched from 10 to 30%, and the S phase cells were not significantly changed after 3 days of induced RNAi (Fig. 4). CRK3 is thus likely playing a similar role in the bloodstream form as in the procyclic form by being an important protein kinase promoting transition of cells across the G2/M checkpoint. The deficiency of CRK2, CRK4, or CRK6 resulted in little appreciable change in the profile of cells compared with the un-induced RNAi cell population (Fig. 4). Taken together, the data indicated little discrepancy in the profiles of CRK functions between the procyclic and bloodstream forms. CRK1 plays a role in catalyzing the G1/S transition, and CRK3 is involved in the G2 to M phase transition in cell cycle control in both forms. The partial growth inhibition from CRK4 deficiency on both forms of T. brucei keeps the possibility open that it could be involved in both G1/S and G2/M transitions.

Distinctive Morphologies of the CRK3-deficient Procytic and Bloodstream Form Cells—The PI-stained CRK1-, CRK3-, and CRK4-depleted cells in both forms were examined under a fluorescence microscope for potential aberrations in intracellular numbers of nuclei and kinetoplasts and cellular morphology. There was no apparent change among the CRK1- and CRK4-depleted cells in both forms compared with the un-induced controls (data not shown). However, when PI-stained CRK3-deficient cells were examined, distinctive morphological changes were observed in the two forms of T. brucei. In the procyclic form 5 days after induction of CRK3 RNAi, the population of one nucleus-one kinetoplast (1N1K) cells was decreased slightly from 75 to 65% (Fig. 5A). But the average size of individual nuclei in the CRK3-deficient 1N1K cells appeared to be enlarged considerably (Fig. 5A) and are designated 1N*1K to be distinguished from the regular 1N1K cells (7). These enlarged nuclei could reflect active DNA synthesis without an accompanied mitosis in the cell cycle. This working hypothesis was verified by the results from another experiment in which BrdUrd incorporation into the DNA of CRK3 knockdown cells was examined in an immunofluorescence assay. The data, presented in Fig. 6, show active BrdUrd incorporation into the enlarged nuclei in a pattern super-imposable with that of the DAPI stain, suggesting that continued DNA synthesis.
may be the primary cause of nuclear enlargement. Formation of the 1N*1K cells may have also contributed to the dramatic increase of G2/M population identified in the FACS analysis (Fig. 2). The 1N2K cells remained at 12–15% of the population following the CRK3 knockdown (Fig. 5B), but the nuclei appeared also significantly enlarged (1N*2K) suggesting a blocked mitosis without an inhibited kinetoplast segregation (data not shown). The population of 2N2K cells was decreased

Fig. 1. Effects of CRK RNAi on the growth rate of procyclic form T. brucei cells. Cloned procyclic trypanosome cells harboring the CRK RNAi plasmid constructs were incubated in culture medium without (+Tet) or with (-Tet) 1.0 μg/ml tetracycline at 26 °C. A, the rate of cell growth was monitored daily, and the cell numbers were plotted in a logarithmic scale. The insets show the intracellular mRNA levels monitored by semi-quantitative RT-PCR from the corresponding RNAi experiments after a 3-day induction. Levels of α-tubulin mRNA (TUB) were measured as a sampling control. B, cells harboring the CRK1 or CRK3 RNAi plasmid were incubated in culture medium without (-Tet) or with (+Tet) 1.0 μg/ml tetracycline at 26 °C. Intracellular mRNA levels of each of the 5 CRKs were monitored by semi-quantitative RT-PCR using gene-specific primers after a 3-day induction. Levels of α-tubulin mRNA (TUB) were measured as a sampling control.
from 12 to 4% without the sign of an enlarged nucleus. These cells were probably formed prior to the loss of CRK3, because there would be no newly formed 2N2K cells under a blocked G2/M transition. The population of anucleated cells, each containing only a single kinetoplast (0N1K, the zoid), was increased from virtual nonexistence to 18% of the population following depletion of CRK3 (Fig. 5B). These could be the daughter cells derived from the division of 1N*2K cells to give rise to 1N*1K cells and the zoids. This postulated capability of an 1N*2K cell to divide suggests that cytokinesis and cell division can be driven and completed by kinetoplast segregation alone without mitosis in the procyclic form.

In the bloodstream form, a 3-day induction of RNAi on CRK3 expression resulted in a significant increase of the 1N2K population from about 14 to 38% (Fig. 7B). This change was accompanied by a corresponding decrease of the 1N1K cell numbers from 72 to 48% of the population (Fig. 7B). The nuclei in these two populations of cells were generally enlarged considerably (1N*1K and 1N*2K) (Fig. 7A), suggesting an arrest of mitosis following a completed S phase in these cells. This suggestion is further reinforced by the observation that BrdUrd was actively incorporated into these enlarged nuclei in 1N*1K, 1N*2K, and XNXK cells in a pattern super-imposable with that of DAPI stain (Fig. 8). One simple explanation for these obser-
vations could be that kinetoplast segregation in the bloodstream form cells proceeds whereas nuclear mitosis is arrested. This would be similar to that observed previously in the procyclic form except that there was virtually no zoid detected in the bloodstream form following the CRK3 knockdown (Fig. 7B). Instead, cells each containing a grossly enlarged nucleus and multiple well segregated kinetoplasts were found to constitute up to 5% of the CRK3-deficient population (Fig. 7). These cells are designated XNXK because shapes of the enlarged nuclei resemble that of a cluster of individual G1 phase nuclei. They are probably not caused by failed initiation of mitosis after repeated rounds of DNA replication but, rather, a failed completion of

![Graphs showing effects of CRK RNAi on the growth rate of bloodstream form T. brucei cells.](image-url)

**Fig. 3. Effects of CRK RNAi on the growth rate of bloodstream form T. brucei cells.** Cloned bloodstream trypanosome cell lines harboring the CRK RNAi plasmid constructs were each incubated in culture medium without (-Tet) or with (+Tet) 1.0 μg/ml tetracycline at 37 °C. The rate of cell growth was monitored daily, and the cell numbers were plotted on a logarithmic scale. The insets show the intracellular mRNA levels monitored by semi-quantitative RT-PCR from the corresponding RNAi experiments after a 3-day induction. Levels of α-tubulin mRNA (TUB) were included as a sampling control.
mitosis. In a case of one 4N4K cell identified in Fig. 7A, there are four separated kinetoplasts and four apparent nuclei linearly chained together. Mitosis was clearly prevented from completion in the four nuclei. But the apparent early emergence of two inseparable nuclei did not prevent the cell from going through another cycle, completing another round of DNA replication and initiating another abortive mitosis resulting in a linear cluster of four nuclei. Meanwhile, the kinetoplast was replicated and segregated twice into four separate kinetoplasts. Unlike the procyclic form, however, this process has apparently failed in driving the cells through cytokinesis and cell division to generate the zoïds in the bloodstream form (Fig. 7). There is apparently a significant distinction in the mechanisms of cell cycle regulation between the two developmental stages of *T. brucei*.

**DISCUSSION**

In our present investigations, we used the data from RNAi experiments to rule out a potential role of CRK2 or CRK6 in regulating the cell cycle progression in either bloodstream or procyclic form of *T. brucei*. CRK4 may play some role in controlling both G1/S and G2/M transitions. But the same data could also suggest a function in promoting cell growth without any involvement with cell cycle regulation. CRK1 clearly plays a role in controlling the G1/S transition in both trypanosome forms. But the partial cell cycle blockade from knocking down CRK1 expression suggests inclusion of other protein kinase(s) in G1/S checkpoint control as well. Homologues of CRK1 have been also cloned from *Trypanosoma cruzi* (TzCRK1) (27) and *Leishmania mexicana* (LmmCRK1) (28). TzCRK1 was shown to
interact with three different \( T. \) \textit{cruzi} cyclins of the PREG1/PHO80 class, \( TzCYC4, -5, \) and -6 (29), whereas \( LmmCRK1 \) was found to be an essential gene in \( L. \) \textit{mexicana} promastigotes (30). Both indications support the notion that CRK1 is a functional CDK involved in regulating G\(_1\)/S transitions among the

![Diagram A: Control vs CRK3 (RNAi)](image)

**Fig. 5.** The morphological phenotypes of CRK3-deficient procyclic form \( T. \) \textit{brucei} cells. The procyclic form \( T. \) \textit{brucei} cells 5 days after CRK3 RNAi induction were stained with PI and examined under a fluorescence microscope. \( A, \) left panel, the control cells in three different types: 1N1K, 1N2K, and 2N2K. \( B, \) right panel, the CRK3-deficient cells in two different types: 1N*1K and 0N1K (zoid). \( B, \) quantification of cells of different morphologies 5 days after CRK3 RNAi induction. Individual cells were scored under a fluorescence microscope by the observable numbers of kinetoplasts (\( K \)) and nuclei (\( N \)). 1N1K and 1N2K include also 1N*1K and 1N*2K, respectively. Data are presented as the mean percent (±S.E.) of total cells counted (>200) from three independent experiments.

![Diagram B: Control vs CRK3 (RNAi)](image)

**Fig. 6.** BrdUrd incorporation into the DNA of CRK3-deficient procyclic form \( T. \) \textit{brucei} cells. BrdUrd was added to the CRK3-deficient procyclic form \( T. \) \textit{brucei} cells 3 days after tetracycline induction, and the cells were harvested 2 days thereafter. Immunofluorescence assays using an anti-BrdUrd monoclonal antibody indicate superimposable patterns between BrdUrd incorporation and DAPI stain in 1N1K and 1N*1K cells.

![Diagram A: Control vs CRK3 (RNAi)](image)

**Fig. 7.** The morphological phenotypes of CRK3-deficient bloodstream form \( T. \) \textit{brucei} cells. The bloodstream form \( T. \) \textit{brucei} cells 3 days after CRK3 RNAi induction were stained with PI and examined under a fluorescence microscope. \( A, \) left panel, the control cells in three different types: 1N1K, 1N2K, and 2N2K. \( B, \) right panel, the CRK3-deficient cells in three different types: 1N2K, XN3K, and 4N4K. \( B, \) quantification of cells of different morphologies 3 days after CRK3 RNAi induction. Individual cells were scored under a fluorescence microscope by the observable numbers of kinetoplasts (\( K \)) and nuclei (\( N \)). 1N1K and 1N2K include also 1N*1K and 1N*2K, respectively. Data are presented as the mean percent (±S.E.) of total cells counted (>200) from three independent experiments.

![Diagram B: Control vs CRK3 (RNAi)](image)

**Fig. 8.** BrdUrd incorporation into the DNA of CRK3-deficient bloodstream form \( T. \) \textit{brucei} cells. BrdUrd was added to the CRK3-deficient bloodstream form \( T. \) \textit{brucei} cells 1 day after tetracycline induction, and the cells were harvested 2 days thereafter. Immunofluorescence assays using an anti-BrdUrd monoclonal antibody indicate superimposable patterns between BrdUrd incorporation and DAPI stain in 1N1K, 1N*1K, 1N*2K, and XN3K cells.

Kinetoplastidae species. A knockdown of CRK1 did not lead to any apparent morphological change in the procyclic form. This is in contrast to the previous observation (11) that depletion of the essential G\(_1\) cyclin CyC1/CYC2 from the procyclic form resulted in formation of slender zoids indicating cell division without a completed S phase. The absence of slender zoids from
the CRK1-depleted population suggests a mere slow down of G1/S transition in these cells, whereas the cell cycle is still capable of progressing eventually to its completion.

CRK3 is apparently the CDK playing an important role in controlling G2/M passage in both bloodstream and procyclic forms of T. brucei. Homologues of CRK3 have been also identified and isolated from T. cruzi (LmmCRK3) (31), L. mexicana (LmmCRK3) (31), and Leishmania major (LmajCRK3) (32), sharing over 75% sequence identities among themselves but only around 50% identity to human CDK1. LmmCRK3 was capable of complementing the Schizosaccharomyces pombe cdc2-33ts mutant, demonstrating that it can carry out the cdc2 function in fission yeast (32). LmmCRK3 has been also shown as an essential gene in L. mexicana (33). By using immunoprecipitation and yeast two-hybrid screen, CRK3 from T. brucei was found associated with the PHO80-like CycE1/CYC2 to form a p12Cks1-binding cyclin-kinase complex and with a mitotic cyclin homologue CycB2/CYC6 to form a p13Cks1-binding complex (12). CycE1/CYC2 was identified by RNAi to be the essential cyclin regulating G1/S transition in procyclic form (11), whereas CycB2/CYC6 was the indispensable cyclin in controlling G2/M passage in both forms of trypanosome (11, 12). These data may agree with the conclusion that formation of a CRK3-CycB2/CYC6 complex is required for initiating passage through the G2/M checkpoint in both procyclic (11) and bloodstream (12) forms of T. brucei. But formation of the CRK3-CycE1/CYC2 complex is apparently not playing an important role in passing the cells across G1/S, because results from the present study showed that a knockdown of CRK3 did not arrest the cells of either form in the G1 phase. The essential function of CycE1/CYC2 in passing the procyclic form cells across G1/S (11) may be thus attributed to a possible complexing with other protein kinase(s), among which CRK1 could be one possibility. Furthermore, our recent study indicated that a knockdown of CycE1/CYC2 expression in the bloodstream form resulted in only a slowing down of the G1/S passage instead of the G1 arrest observed in the procyclic form (11). This discrepancy suggests that there may also be additional cyclins that form complexes with yet other unidentified CDKs involved in the G1/S passage in bloodstream form. Further investigations will be required to clarify the two different profiles of cyclins and the other CDKs involved in G1/S checkpoint regulations in the two developmental forms of trypanosome.

Another interesting observation from our present study is that the knockdown of CRK3 in both T. brucei forms exerted no apparent effect on the kinetoplast segregation. In procyclic form the continued kinetoplast segregation led to zoid formation with the nucleus remaining in the other 1N+1K daughter cell approximately twice of the size as that in the G1 cell (Fig. 5A and Fig. 6). There was no apparent formation of grossly enlarged, irregularly shaped multiple nuclear aggregates. This suggests the following: (a) cytokinesis and cell division can be driven by kinetoplast segregation and proceed to completion when the G2/M transition is blocked in the procyclic form; (b) but completion of this “aborted” cell division prevents the 1N+1K cell from a subsequent re-entry into another round of S phase. A similar phenotype was also observed in the CycB2/CYC6 knockdown in the procyclic form (11, 12). In the bloodstream form, however, kinetoplast segregation can proceed for at least two or more rounds in the absence of CRK3. But it fails to bring about cytokinesis or cell division. Remarkably, the absence of cell division does not prevent the cell from progressing through G1 and re-entering into another S phase resulting in four partially divided, albeit still inter-connected, nuclei (Fig. 7A). A similar observation was also made from a knock-down of CycB2/CYC6 in the bloodstream form, albeit no multiple nuclear aggregates have yet been reported (12). This apparent lack of a checkpoint between mitosis and S phase in the bloodstream form of T. brucei provides yet another example of the unusually simple as well as leaky cell cycle regulation in trypanosome.

In another recent study (34), an RNAi of the expression of a glycosylphosphatidylinositol GPI-linked protein transaminase gene GPI8 in T. brucei resulted in no detectable effect on the in vitro growth of procyclic form but caused death of the bloodstream form with a defined multinuclear, multikinetoplast, and multiflagellar phenotype indicative of a failed cell division, whereas kinetoplast segregation, DNA replication, and mitosis continued to proceed through many rounds. By assuming that depletion of GPI8 brings about inhibition of cytokinesis in the trypanosome, it apparently does not inhibit cell division in the procyclic form, but inhibits division of the bloodstream form without affecting the cell cycle progression. The data are thus in good agreement with our current findings.

The many distinctions between T. brucei procyclic and bloodstream forms in the mechanisms of cell cycle regulation have not yet, to our knowledge, found a parallel among other eukaryotic species. Some of these distinctions, such as the apparently different G1 cyclins involved in controlling G1/S transition, could be attributed to the changing living environments of the trypanosome, making it necessary for the organism to respond to different external triggers for cell cycle control. However, it is somewhat more difficult to explain why, after kinetoplast segregation without a completed mitosis, cytokinesis and cell division would occur in the procyclic form but not in the bloodstream form. At a closer examination of the cellular ultrastructures of the two forms, however, the kinetoplast is found located at the very posterior end of bloodstream form cell but to a location midway between the nucleus and the posterior end of the procyclic form cell (35). Due to the elongated cellular morphology of the latter, the changed location of kinetoplast was postulated to be a necessity for achieving the bilateral symmetry required for cell division (6). Whether the changed location of kinetoplast could make a kinetoplast-driven cell division possible without completing the mitosis remains to be elucidated.

Cytokinesis in the procyclic form can be inhibited by okadaic acid, a protein phosphatase inhibitor, resulting in cells with multiple (and well separated) nuclei and a single kinetoplast (10). Similarly, an RNAi silencing of the expression of dynein heavy chain 1b or intraflagellar transporter 88 in the procyclic form led to a loss of flagellum and a failure to undergo cytokinesis (36), which also resulted in multinucleated cells. The procyclic form is thus similar to the bloodstream form in that the progression of nuclear cell cycle continues when cytokinesis (and cell division) is inhibited. This breakdown of coordination between cytokinesis, which is probably driven primarily by kinetoplast segregation, and nuclear cell cycle progression may represent one of the most intriguing biological phenomena likely shared by all members of the Kinetoplastida family due to the presence of a single mitochondrion in each cell. The family may provide a useful model for our further understanding of the mechanisms initiating cytokinesis in eukaryotes.

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X. Tu and C. C. Wang, manuscript in preparation.
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The Involvement of Two cdc2-related Kinases (CRKs) in Trypanosoma brucei Cell Cycle Regulation and the Distinctive Stage-specific Phenotypes Caused by CRK3 Depletion

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