The Trypanosoma brucei Cyclin, CYC2, Is Required for Cell Cycle Progression through G1 Phase and for Maintenance of Procyclic Form Cell Morphology*

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CYC2 is an essential PHO80-like cyclin that forms a complex with the cdc2-related kinase CRK3 in Trypanosoma brucei. In both procyclic and bloodstream form T. brucei, knock-down of CYC2 by RNA interference (RNAi) led to an accumulation of cells in G1 phase. Additionally, in procyclic cells, but not in bloodstream form cells, CYC2 RNAi induced a specific cell elongation at the posterior end. The G1 block, as well as the posterior end elongation in the procyclic form, was irreversible once established. Staining for tyrosinated α-tubulin and morphometric analyses showed that the posterior end elongation occurred through active microtubule extension, with no repositioning of the kinetoplast. Hence, these cells can be classified as exhibiting the “nozzle” phenotype as has been described for cells that ectopically express TbZFP2, a zinc finger protein that is involved in the differentiation of the bloodstream form to procyclic form. Within the tsetse fly, procyclic trypanosomes differentiate to elongated mesocyclic cells. However, although mesocyclic trypanosomes isolated from tsetse flies also show active microtubule extension at the posterior end, the kinetoplast is coincidentally repositioned such that it always lies approximately midway between the nucleus and posterior end of the cell. Thus, in the procyclic form CYC2 has dual functionality and is required for both cell cycle progression through G1 and for the maintenance of correct cell morphology, whereas in the bloodstream form only a role for CYC2 in G1 progression is evident.

The African trypanosome, Trypanosoma brucei, is the causative agent of sleeping sickness in humans and Nagana in cattle. T. brucei, a unicellular protozoan parasite, exhibits a complex biphasic life cycle split between the tsetse fly and mammalian hosts (1, 2). The life cycle is characterized by a series of differentiation steps resulting in a number of morphologically, structurally, and biochemically distinct forms. In addition, the parasite alternates between replicating and nonreplicating forms, indicating that there is an integral link between differentiation and cell cycle control. Further, the position of the kinetoplast organelle (which contains the DNA of the organism’s single mitochondrion) relative to the nucleus and posterior end of the cell alters with the different developmental forms and may play a pivotal role in controlling differentiation (3).

Previous work has shown that the T. brucei cell cycle is likely to be complex and to display distinct features compared with other unicellular eukaryotes (4). For example, discrete phases for the replication and segregation of nuclear and kinetoplast DNA have been described (5). Cells in G1 phase of the cell cycle have one nucleus and one kinetoplast. Kinetoplast S phase commences prior to nuclear S phase, is of a shorter duration, and therefore completes before nuclear S phase finishes. Early in G2 phase, the replicated kinetoplast segregates, resulting in a cell with one nucleus and two kinetoplasts. Mitosis occurs, resulting in a cell with two nuclei and two kinetoplasts, before cytokinesis occurs and cells reenter into G1. Additionally, some eukaryotic cell cycle checkpoints, such as the mitosis to cytokinesis checkpoint, are absent in trypanosomes (6, 7).

The role of cyclin-dependent kinases in the eukaryotic cell cycle is well established, and a number of cell cycle control proteins have been identified in T. brucei. Seven putative cdc2-related kinases (CRKs) and eight cyclins have been described (8). Based on sequence similarity to other known cyclins, the T. brucei cyclins can be grouped into three classes: the PREG1/PHO80-like cyclins (CYC2, CYC4, CYC5, and CYC7), the mitotic-like cyclins (CYC3, CYC6, and CYC8), and the transcriptional cyclins (CYC9). It is thought that CRK3 is the functional trypanosome CDK1 (8), and this kinase has been shown to interact with two trypanosome cyclins, CYC2 (9) and CYC6 (7) to form active kinase complexes in vivo. CRK3-CYC6 is required for progression through the G2/M phase boundary into mitosis, but the function of CRK3-CYC2 remains undefined. The cyclin box homology region of CYC2 exhibits 38% identity to PREG1 from Neurospora crassa and 25% identity to PHO80 from Saccharomyces cerevisiae at the amino acid level (9). PHO80 is one of 10 cyclins that bind to the CDR PH085. The PH085 cyclins can be split into two functional classes, the PHO80 and the PCL1,2 subfamilies. PH080 subfamily members play regulatory roles in nutrient metabolism (10). For instance, PH080 complexed with PH085 regulates phosphate metabolism through regulation of the PHO4 transcription factor (11). PREG1 plays an analogous role in N. crassa (12). Members of the PCL1,2 subfamily play roles in cell cycle progression through Start (10, 13), morphogenesis (14), and possibly in controlling the stability of the transcription factor GCN4 (15). Because the cyclin moiety of a CDR complex confers

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‡ The abbreviations used are: CRK(s), cdc2-related kinase(s); CYC, cyclin; DAPI, 4,6-diamidino-2-phenylindole; N, nucleus; K, kinetoplast; RNAi, RNA interference; RT, reverse transcription.
functional specificity (16), it seems possible that CKK3-CYC2 plays a regulatory role in nutrient metabolism in trypanosomes. In this paper we use RNA interference (RNAi) to investigate the role of CYC2 in *T. brucei*. Although we do not rule out a role for CYC2 in nutrient metabolism, we demonstrate that it carries out functions that could not be predicted from primary sequence analysis, revealing that it regulates both cell cycle progression and cell morphology in the African trypanosome.

### EXPERIMENTAL PROCEDURES

**Recombinant DNA Techniques**—Standard DNA techniques were performed as described in Ref. 17. Plasmid isolation from *Escherichia coli* and DNA extraction from agarose gels used Qiaprep and Qiaex kits from Qiagen, respectively. DNA sequencing was performed by the MBSU DNA sequencing service (University of Glasgow) using an ABI automatic sequencer.

**Transfection of *T. brucei***—Culture and transfection of *T. brucei* were carried out essentially as described previously (18). Where necessary, hygromycin was added at a concentration of 50 μg ml\(^{-1}\) (procyclic cells) or 5 μg ml\(^{-1}\) (bloodstream form cells), G418 at 10 μg ml\(^{-1}\) (procyclic cells) or 2.5 μg ml\(^{-1}\) (bloodstream form cells), zeocin at 10 μg ml\(^{-1}\) (procyclic cells), and phleomycin at 2.5 μg ml\(^{-1}\) (bloodstream form cells).

**CYC2 RNAi Cell Lines**—For RNAi, the procyclic 427 pLew13 pLew29 and the bloodstream form 427 pLew13 pLew90–6 cell lines (19) were transfected with plasmid pGL598. To generate pGL598, a BamHI-XhoI fragment of CYC2 comprising the last 485 bp of the CYC2 sequence was excised from pGL256 (generated by subcloning an XhoI–EcoRI fragment incorporating the last 485 bp of the KCN2 sequence into the BamHI–EcoRI sites of pGL256) and cloned into the BamHI–XhoI sites of pBlueScript SK– (Stratagene) and used to replace the BamHI-XhoI GFP fragment of p2T7ti/GFP (20). Independent clones were generated by limiting dilution cloning. To induce RNAi in procyclic forms, the cell line was grown to a density of 5 × 10\(^5\) cells ml\(^{-1}\), diluted to give a density of 5 × 10\(^4\) cells ml\(^{-1}\), and cultured in the presence or absence of 50 μg ml\(^{-1}\) tetracycline. Cells were counted daily using a Neubauer improved hemocytometer, and cultures were diluted to 10\(^5\) cells ml\(^{-1}\) when the density approached 10\(^5\) cells ml\(^{-1}\). Bloodstream form cells were induced at a density of 10\(^5\) cells ml\(^{-1}\) with 1 μg ml\(^{-1}\) tetracycline and were diluted back to 10\(^5\) cells ml\(^{-1}\) whenever the cell density approached 10\(^6\) cells ml\(^{-1}\). To reverse the RNAi, cultures that had been incubated with tetracycline were washed twice in phosphate-buffered saline or trypanosome dilution buffer (5 mM KCl, 80 mM NaCl, 1 mM MgSO\(_4\), 20 mM EDTA, pH 7.4) before being resuspended in fresh medium without tetracycline. Total RNA and first strand cDNA were generated from cells exactly as described previously (17). Multiplex PCRs were set up, using 1 μl of DNA/reaction and PIGO primers (OL827 and OL828) or GFP primers (OL565 and OL566) plus CYC2 primers (OL171 and OL1079) or CYC6 primers (OL827 and OL828) plus CYC2 primers (OL827 and OL828) or CYC6 primers (OL827 and OL828) for all PCR reactions. PCR products were purified and cloned into pBluescript SK– (Stratagene). The sequences of oligonucleotides used in this study are given in Table I. PCRs contained 45 mM Tris–HCl, pH 8.8, 11 mM NH\(_4\)SO\(_4\), 4.5 mM MgCl\(_2\), 6.7 mM β-mercaptoethanol, 4.4 μM EDTA, pH 8.0, 10 mM dNTPs, 113 μM 5′-bovine serum albumin, 50 μM each primer, 1 μl of cDNA, and 1 unit of Taq DNA polymerase (ABgene). The conditions for PCR were 1 cycle of 95°C for 30 s, 30 cycles of 95°C for 50 s, 50°C for 5 s, 72°C for 1 min 30 s, and 1 cycle of 72°C for 5 min.

**p12\(^{2/3}\)- and p12\(^{1/3}\)-binding Kinase Assays**—Leishmania p12\(^{2/3}\) or yeast p12\(^{1/3}\) protein was coupled to Amino-link beads (Pierce) at a concentration of 5 mg ml\(^{-1}\) as described previously (21). Amino-link beads coupled to Tris–HCl, pH 7.4, to block the reactive sites were used as a negative control. S100 lysates (prepared as described previously (9)) were incubated with 50 μl of control, p12\(^{2/3}\) or p12\(^{1/3}\) beads for 1 h at 4°C. Beads were washed extensively with lysis buffer and used for histone H1 kinase assays as described previously (9).

**Immuno blotting**—Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane. Western blots were performed as described previously (21) with a 1:10,000 dilution of a mouse monoclonal anti-EF-1α antibody (clone CBP-K1, Upstate Biotechnology, Lake Placid, NY) followed by a 1:10,000 dilution of goat anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody (Promega). The West-Dura chemiluminescence detection system (Pierce) was used to visualize antigens.

**DAPI Staining and Flow Cytometry**—DAPI staining of methanol-fixed cells and analysis of propidium iodide–stained cells by flow cytometry were carried out exactly as described previously (7). Using the CellQuest version 3.3 software, each of the three peaks (representing cells in G\(_1\), S, and G\(_2\)/M phases, respectively) obtained in the flow cytometry profile of fluorescence plotted against cell number was gated and then replotted as a function of forward scatter. Because a population of cells was analyzed in this way, the forward scatter can be taken to be directly proportional to cell size.

**Immunofluorescence and Morphometry**—For immunofluorescence, procyclic form *T. brucei* was fixed in suspension in 4% paraformaldehyde overnight at 4°C, washed twice in 2 ml of phosphate-buffered saline, and resuspended in phosphate-buffered saline. To permeabilize trypanosomes, fixed cells were incubated in 500 μl of 0.1% Na\(_2\)HPO\(_4\) and 0.1% Triton X-100, for 15 min and then 1 ml of 0.05 M Na\(_2\)HPO\(_4\) with 0.1% Triton X-100, for 5 min. EP procyclin was detected by incubating cells with the mouse monoclonal antibody RP-1 (CedarLane) diluted 1:500 in phosphate-buffered saline, 1% bovine serum albumin. The Alexa Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes, 1:1000) was used as the secondary antibody. Tyrosinated α-tubulin was detected with the rat monoclonal antibody YL1/2 (20) (1:50) and CY3-conjugated goat anti-rat secondary antibody (Amersham Biosciences) (1:1,000). Nuclear and mitochondrial DNA was stained with DAPI. Mesocyclic trypanosomes were methanol fixed at −20°C overnight before being stained with the YL1/2 antibody and DAPI. Image acquisition was performed with a motorized Zeiss Axiophot 2 widefield microscope equipped with a CCD camera and integrated using the scripting features of the IPLab for Macintosh software (version 3.5, Scanalytics, Fairfax, VA; for details, see http://www.scanalytics.com/).

For morphometric analyses, three-channel images of randomly chosen, nonoverlapping areas of the specimen were acquired, segmented, and measured using an IPLab software script. Cells were identified by threshold segmentation using procyclin cell surface fluorescence. The distances between the posterior end of the cell and the kinetoplast, between kinetoplast and nucleus, and between nucleus and the anterior end of the cell, respectively, were measured by applying the line measurement tool from the IPLab software to the merged three-channel fluorescent images. Scoring for tyrosinated α-tubulin was carried out for all cells measured using a fluorescence intensity threshold. Final results are given as means ± S.D. of n = 65–83 cells/time point and cell line. The analysis of 1N2K and 2N2K cells for noise formation was essentially performed as described above, except for the DAPI channel segmentation. All cells showing fewer than three DAPI-positive objects were excluded from analysis. Scoring for 1N2K or 2N2K configurations was done by inspection of the final segments.

### RESULTS

**RNAi of CYC2 in Procyclic Trypanosomes**—To investigate the role of CYC2, an RNAi approach was taken. A fragment of CYC2 was cloned into vector p2T7ti (20) and transfected into the *T. brucei* procyclic 427 pLew13 pLew29 cell line (19). Two
FIG. 1. RNAi of CYC2 in procyclic form cells. A, growth curves. RNAi cell lines were grown in the presence or absence of tetracycline, and the cell number was monitored over time. Representative cumulative growth curves for each procyclic CYC2 RNAi clone are shown. Filled triangles, clone C3; tetracycline; open triangles, C3 + tetracycline; filled circles, clone D3; tetracycline; open circles, D3 + tetracycline. B, Southern blots of RT-PCRs for procyclic clone D3. RNA was prepared from cells after a 24-h induction. First strand cDNA was generated, and multiplex PCRs were carried out using PIGO or GPI8 primers with either CYC2 or CYC6 primers. Products were blotted onto membrane and visualized by probing with the relevant genes. tet, tetracycline; RT, reverse transcription; PIGO, GPI8. C, kinase assays for CYC2 RNAi cell lines. Uninduced and induced cell lysates (120 h, 1 × 10^6 cell equivalents) were incubated with control, p12, or p13 beads before being assayed for histone H1 kinase activity. Results are shown for procyclic clone D3. D, Western blot. CYC2 RNAi clone D3 was grown in the absence or presence of tetracycline for 120 h before cell lysates were prepared and analyzed by SDS-PAGE (10^6 cell equivalents/lane) followed by Western blotting with anti-EF-1α antibody.

Fig. 2. Analysis of procyclic CYC2 RNAi cells. A, nucleus and kinetoplast configurations (clone D3) as determined by DAPI staining over time. B, images of procyclic CYC2 RNAi cells. Cells (clone D3) were stained with DAPI (from left to right: phase-contrast, DAPI-stained, and overlay). Top, uninduced; bottom, tetracycline (tet)-induced (120 h).

To demonstrate that RNAi of CYC2 led to a decrease in CYC2 kinase activity, cell lysates of CYC2 RNAi cell lines were incubated with beads conjugated to leishmanial p12^{kd-1} (21) or yeast p13^{uc-1} (23) before being assayed for histone H1 kinase activity (Fig. 1C). Previously we have shown that several active T. brucei CRK complexes (including CRK3-CYC2) bind to p12^{kd-1} beads (9), whereas CRK3-CYC6 (the G₂/M phase kinase complex) binds only to p13^{uc-1} (7). Induction of CYC2 RNAi led to a significant reduction in p12^{kd-1}-binding kinase activity after a 120-h induction, although it appeared that other p12^{kd-1}-binding kinases remained active. Interestingly, most of the p13^{uc-1}-binding kinase activity was lost following induction of CYC2 RNAi, suggesting that CRK3-CYC6 was not active in these cells. To demonstrate that this was not the result of a general degradation of cellular protein, cell extracts prepared from CYC2 RNAi cells induced for 120 h were shown to have equivalent amounts of the housekeeping protein EF-1α compared with uninduced cells (Fig. 1D).

The reason for the observed growth arrest was investigated. The nucleus and kinetoplast configurations of CYC2 RNAi cells were monitored over time by staining cells with DAPI. Induction of CYC2 RNAi in procyclic trypanosomes resulted in almost 100% of cells exhibiting a one-nucleus, one-kinetoplast (1N1K) configuration after 144 h (Fig. 2A). Morphological differences between uninduced and induced cells were also seen by phase-contrast microscopy (Fig. 2B), with induced cells exhibiting significant elongation at the posterior end. To confirm that these 1N1K cells were in G₁ phase of the cell cycle, CYC2 RNAi cells were subjected to DNA content analysis by flow cytometry (Fig. 3A). Induction of CYC2 RNAi for ~4 days resulted in an increase in the number of cells with a 2C DNA content (normal G₁ DNA content), and a concomitant decrease in cells with greater than 2C DNA content. Additionally, no cells were seen with abnormally high DNA contents (greater than 4C), indicating that these cells did not re-replicate their DNA following the cell cycle arrest. Taken together with the p12^{kd-1} and p13^{uc-1}-binding kinase activity data described above, these data suggest that CYC2 RNAi results in a buildup
of cells in G1 phase of the cell cycle which are unable to enter S phase. Because cells accumulate in G1, this is consistent with the loss of p13suc1-binding (G2/M phase) kinase activity observed following induction (Fig. 1C).

The relative sizes of cells from different cell cycle phases in procyclic CYC2 RNAi cells were also assessed. The G1, S, and G2/M phase cell populations seen in the flow cytometry profiles of fluorescence plotted against cell number were gated, and cells from these populations were replotted as forward scatter (proportional to cell size) versus number. Top, uninduced; bottom, tetracycline-induced (119 h).

The morphological changes occurring following induction of CYC2 RNAi were examined in more detail. Superficially, cells induced for CYC2 RNAi resembled those seen upon ectopic expression of TbZFP2 in procyclic cells, which elongate specifically at the posterior end, resulting in an extended kinetoplast to posterior end dimension, a phenotype termed “nozzle” (24). This posterior end elongation can be visualized by staining cells with the YL1/2 antibody that recognizes tyrosinated α-tubulin present in newly assembled microtubules. In cells with normal morphology, YL1/2 stains basal bodies, newly growing flagella, and the posterior tip. In nozzle cells, the staining at the posterior end of the cell is unusually bright because of the extension of the posterior microtubules.

To investigate whether CYC2 RNAi cells had the dimensions and microtubule extension consistent with the nozzle phenotype, cells were harvested at 24-h intervals following induction of CYC2 RNAi, fixed, and triple stained with an anti-procyclin antibody (to visualize the surface of the cells), the YL1/2 antibody and DAPI, to visualize nuclei and kinetoplasts (Fig. 4). The nucleus to anterior tip, the nucleus to kinetoplast, and the kinetoplast to posterior tip dimensions were measured using the IPLab 3.5 software (Fig. 5A). The parental RNAi cell line 427 pLew13 pLew29, induced with tetracycline under the same conditions as the CYC2 RNAi cell line, was included in the analyses as a control. Cells were present with an extended kinetoplast to posterior end dimension when compared with uninduced cells for all configurations (1N1K, 1N2K, and 2N2K) (Figs. 4 and 5). For the majority of these elongated cells, YL1/2 antibody staining localized to this extended posterior tip region, indicating that active microtubule extension occurred. In some cases, the YL1/2 staining localized to a subposterior end region (such as for the 2N2K cell in Fig. 4B), rather than to the very tip of the cell, but in all cases, the staining appeared posterior to the kinetoplast. Additionally, some cells that showed nozzleing by morphometry did not exhibit extensive YL1/2 staining at the posterior end, possibly because of them having stopped actively extending and therefore no longer staining with YL1/2. The percentage of nozzleled cells of a 1N1K configuration increased over time, such that by 96 h postinduction, 100% 1N1K cells could be classed as nozzles through morphometry. The percentage of 1N1K cells that also stained positively at the posterior end with YL1/2 reached a maximum of 80% at 96 h but was reduced to around 50% by 144 h postinduction. Although by 96 h postinduction of CYC2 RNAi, all 1N1K cells were classified as nozzles by morphometry, maximally 50% of 1N2K and 2N2K cells could be classified as nozzles by 120 h postinduction (Fig. 5C). However, although only 50% of 1N2K and 2N2K cells formed nozzles, virtually all cells of these configurations by 96 h postinduction displayed abnormal kinetoplast positioning. Interestingly, in all 1N1K cells, where basal body staining was not masked by the intensity of the YL1/2 staining at the posterior end, only one basal body was present (Fig. 4C), indicating that these cells were arrested in the cell cycle before the point of basal body duplication.

It has been reported that during trypanosome development in the tsetse fly, procyclic form trypanosomes differentiate to mesocyclic form trypanosomes, which are elongated and arrested in G0/G1 phase of the cell cycle (2). To determine whether induced CYC2 RNAi procyclic cells resembled mesocyclic trypanosomes, mesocyclic trypanosomes dissected from the proventriculus of an infected tsetse fly were examined by microscopy. Cells were stained with the YL1/2 anti-tyrosi-
form to the mesocyclic form is reversible, but the induction of a nozzle phenotype is irreversible.

**RNAi of CYC2 in Bloodstream Form Trypanosomes**—To determine whether RNAi of CYC2 would lead to the same phenotype in bloodstream form cells, the CYC2 RNAi construct was transfected into cell line 427 pLew13 pLew90–6 (19), and three independent clones were selected for further analysis. RNAi of CYC2 in bloodstream form cells resulted in a decreased growth rate apparent from 24 h postinduction (Fig. 7A) that was associated with a specific reduction in CYC2 mRNA as determined by RT-PCR (Fig. 7B). Although a significant reduction in CYC2 mRNA occurred relative to the PIGO loading control, CYC6 mRNA levels remained approximately constant. Each independent clone gave essentially the same phenotype, so for this and subsequent analyses, only the data for one clone are shown.

Induction of CYC2 RNAi in bloodstream form trypanosomes resulted in an increase in cells exhibiting a 1N1K configuration, although the percentage of cells with a 1N1K configuration never exceeded 90% (Fig. 8A). Morphological differences between uninduced and induced cells as seen by phase-contrast microscopy in the procyclic CYC2 RNAi cells were not observed in the bloodstream form (data not shown). Flow cytometry of induced bloodstream form CYC2 RNAi cells revealed a significant decrease in cells with greater than 2C DNA content by 4 days postinduction and a significant increase in the number of cells with 2C DNA content by 5 days postinduction (Fig. 8B). Cells with DNA contents of greater than 4C were not seen. These data suggest that CYC2 RNAi in the bloodstream form, as in the procyclic form, results in an accumulation of cells in G1 phase of the cell cycle.

To confirm that RNAi of CYC2 in the bloodstream form did not result in any significant changes in cell size, the relative sizes of cells from different cell cycle phases were assessed. The G1, S, and G2/M phase cell populations seen in the flow cytometry profiles of fluorescence plotted against cell number were gated, and cells from these populations were replotted as forward scatter against cell number (Fig. 8C). Analysis of uninduced cells revealed that S phase cells are larger than G1 phase cells, and G2/M phase cells are larger than S phase cells, indicating that bloodstream form trypanosomes grow through S phase and G2/M phase. Analysis of CYC2 RNAi-induced bloodstream form cells showed that these cells are not significantly larger in size than their uninduced counterparts, confirming microscopic observations that these cells do not exhibit significant morphological changes. Removal of tetracycline from the medium after 48 h allowed the population to resume a normal growth rate around 2 days later, whereas removing tetracycline from the medium after 96 h did not restore normal growth rates (data not shown).

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**Fig. 4. Microscopy of procyclic CYC2 RNAi cells.** Cells were triple stained with anti-procyclin (green), anti-tyrosinated α-tubulin (red), and DAPI (blue). A, comparison of uninduced (left) and tetracycline (tet)-induced (right) cells. B, comparison of induced cells of 1N1K, 1N2K, and 2N2K configurations. Upper image, triple overlay; lower image, YL1/2 and DAPI overlay. C, dual stained 1N1K cells (anti-tyrosinated α-tubulin and DAPI). Arrows indicate the position of the single basal body (BB), nucleus (N), and kinetoplast (K) in each cell.

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**DISCUSSION**

We have shown using RNAi that knock-down of CYC2 in procyclic or bloodstream form trypanosomes leads to an accumulation of cells in G1 phase of the cell cycle as determined by both the nucleus and kinetoplast configuration of cells and flow cytometry. Additionally, we show that induced CYC2 RNAi cells have the biochemical characteristics of G1 phase cells because they display greatly reduced activity of the mitotic kinase CRK3-CYC6. Further, staining of induced CYC2 RNAi procyclic cells with the YL1/2 antibody stained only one basal body/1N1K cell (Fig. 4C), providing evidence that these cells are in G1 phase (5). Therefore, from these data, we conclude that CYC2 is required for progression through G1 phase. It is possible that CYC2 forms part of the molecular machinery that determines whether the cell is ready to enter a new cell cycle or that it is required for entry into S phase.

Interestingly, the cell cycle arrest seen with RNAi of CYC2...
appears earlier in the cell cycle than that seen previously with either aphidicolin or acriflavine treatment of procyclic trypanosomes (6, 25). Aphidicolin inhibits nuclear DNA synthesis but not kinetoplast DNA synthesis. Treatment of procyclic trypanosomes with aphidicolin leads to an accumulation of cells in S phase, and at later time points 1N2K cells (with incompletely replicated nuclear DNA) divide, without undergoing mitosis, to give rise to a 1N1K daughter and a zoid (0N1K) (6). Acriflavine treatment of procyclic trypanosomes selectively inhibits kinetoplast replication (25), but basal body duplication and segregation are unaffected. The single kinetoplast associates with one of the two basal bodies (in the majority of cases, with the basal body subtending the old flagellum), and the cell cycle continues, leading to a cell division that results in asymmetric segregation of kinetoplast DNA, with the formation of a 1N1K daughter and a dyskinetoplastic cell (1N0K). Over time, CYC2 RNAi cells are prevented from entering S phase (either nuclear or kinetoplast) and stop dividing. Basal body duplication and segregation also appear inhibited, with the result that neither procyclic nor bloodstream form CYC2 RNAi trypanosomes are competent for cell division.

In both bloodstream and procyclic trypanosomes, CYC2 mRNA was essentially ablated 24 h after induction of the RNAi. Based on a half-life of less than 9 h (26), and taking into account cell doublings, knock-down of ~90% of CYC2 protein would be expected by 24 h postinduction, and less than 1% CYC2 protein would be expected to remain by 48 h postinduction. However, despite a similar penetrance of the RNAi in both life cycle stages, absence of CYC2 led to a total G1 cell cycle block and growth arrest by 1 week following induction of the RNAi in procyclic cells, whereas in bloodstream form cells, only an enrichment of cells in G1 phase, up to a maximum of ~90% of the population was seen after a similar induction period,
Our results differ from those reported recently by Li and Wang (27), who found that induction of CYC2 RNAi in procyclic trypanosomes resulted in a growth arrest, which was accompanied by an increase in 1N1K cells and zoids. Although we observed previously the generation of zoids in procyclic form trypanosomes following CYC6 RNAi, as a result of a block in progression of mitosis and the absence of a checkpoint that prevents initiation of cytokinesis in the absence of completion of mitosis (7), we never observed an increase in zoids in CYC2 RNAi cell lines. The difference in observed phenotype may reflect differing levels of penetrance between the cell lines of the two groups, possibly as a result of differences in growth conditions and medium, or of using different RNAi vectors. If, for instance, the level of knock-down of CYC2 is not sufficient to prevent initiation of S phase (kinetoplast and/or nuclear) in all cells but is sufficient to prevent completion of S phase, then this subset of cells will divide as 1N2K cells to give a 1N1K daughter and a zoid (as seen with aphidicolin treatment described above).

Our results further demonstrate that CYC2 is essential for the maintenance of procyclic form cell morphology, although we cannot determine whether this is a direct function of CYC2 or whether its absence triggers downstream events that alter cell morphology. This is the first definitive demonstration of a CRK cyclin complex playing dissimilar roles in different life cycle stages and may indicate that separate cell cycle regulatory mechanisms operate to allow for differences in morphology and environment of these two life cycle stages. Absence of CYC2 in the procyclic form led to the formation of cells with a nozzle phenotype, whereby the kinetoplast to posterior end dimension of the cell was specifically elongated. This phenotype has been described previously for cells ectopically expressing the CCCH zinc finger protein TbZFP2 (24). However, it is not yet clear whether these incidences of nozzle formation occur through the same or independent pathways. It is conceivable that CYC2 could function upstream of TbZFP2 in a differentiation pathway that controls posterior end extension in vivo, and in the case of CYC2, which has sequence similarity to PHO80-type cyclins, one could speculate that this molecule is regulated by environmental cues to bring about the morphological changes of differentiation. In the tsetse fly, the differentiation of the procyclic form to the mesocyclic form is accompanied by cell elongation, which we have shown by staining for tyrosinated α-tubulin occurs through active extension of microtubules at the posterior end of the parasite. It would prove interesting to determine whether CYC2-associated kinase activity is also down-regulated in the mesocyclic form relative to the procyclic form. Little is known about the mesocyclic insect stage, with classification of this form relying largely on morphology and location in the tsetse fly (2). This work therefore provides the first cytological marker for the mesocyclic trypanosome. Further, we have shown by DAPI staining that the kinetoplast in mesocyclic trypanosomes is positioned midway between the nucleus and posterior tip of the cell. Taken together, this suggests that the kinetoplast is repositioned coincidently with the posterior end extension during the procyclic to mesocyclic differentiation step in the tsetse fly to maintain equal kinetoplast to posterior end and kinetoplast to nucleus dimensions. However, because procyclic cells form nozzles under certain conditions, posterior end elongation and kinetoplast repositioning must be separable events.

It is clear that CYC2 plays a bifunctional role in procyclic form trypanosomes. In procyclic CYC2 RNAi cells, the onset of the growth arrest and G1 block became apparent from 72 to 96 h postinduction (Figs. 1 and 2). However, the onset of the increasing posterior end to kinetoplast dimension was visible...
from 48 to 72 h in cells of all NK configurations (Fig. 5). This therefore suggests that in the population as a whole, commencement of nozzle formation precedes the G₁ block. This may indicate that the G₁ block in procyclic cells is dependent upon the onset of nozzle formation or that a greater reduction in CYC2 levels is required to achieve a G₁ block compared with the bloodstream form. The same is also true for procytic trypanosomes. Cell growth has been reported in all cell cycle phases except M phase for both yeast and mammalian cells (29). Yeast control their cell size by means of cell size checkpoints operating in G₁ and G₂, ensuring that progression through the cell cycle is delayed if the cell has not obtained a critical threshold size (30). Although for many years, it has been assumed that similar control mechanisms operate in mammalian cells, recent work has suggested that mammalian cells are in fact more likely to control their cell size through the independent but coordinated control of cell division and growth by a range of stimuli (29, 31). Therefore, the lack of growth during G₁ phase sets T. brucei apart from both yeast and mammalian cells, and it will prove interesting to discover whether a cell size checkpoint exists in this organism.

Our work has shown that the trypanosome cyclin CYC2 is required for cell cycle progression through G₁ and for the maintenance of procyclic cell morphology, although we cannot rule out additional regulatory roles in nutrient metabolism. The functions we have described are at odds with the classification of CYC2 and HO80-like cyclins through sequence similarity and are more reminiscent of the budding yeast PCL1,2 subclass of PHO85 cyclins (10). Deletion of all five members of the PCL1,2 subfamily in S. cerevisiae mimics the morphogenetic phenotype seen upon PHO85 deletion, resulting in cells with elongated buds and a wider bud neck (14). Actin localization is also abnormal in these cells and leads to random patterns of budding (32, 33). Expression of the PCL1 and PCL2 cyclins (required for passage through Start) peaks in late G₁, whereas PCL9 expression peaks in late M phase/eary G₂ phase (10, 13). In contrast, the remaining PCL1,2 cyclins, CLG1 and PCL5, are expressed constantly through the cell cycle.

In mammals, progression through G₁ phase is regulated by CDK4 and CDK6 complexed with cyclins D1-D3 and by CDK2-cyclin E (34, 35). D-type cyclins are expressed in response to mitogenic stimulation (36, 37), whereas cyclin E mRNA and protein accumulate in late G₁ peak at the G₁/S phase transition and are down-regulated through degradation by the ubiquitin-proteosome system during S phase (38, 39). Overexpression of the D-type cyclins or cyclin E during early G₁ results in premature entry into S phase (40). Despite CYC2 being degraded by the proteosome system (26), CYC2 bears no significant sequence similarity to human cyclin E, and currently data on the expression pattern of CYC2 throughout the trypanosome cell cycle are not available. Additionally, we have overexpressed CYC2 as a TY-tagged fusion protein in procyclic T. brucei and observed no obvious growth or morphological effects or significant changes in flow cytometry profile. CYC2 appears unique among G₁ cyclins in that it also regulates morphogenesis. It seems that T. brucei has adapted evolutionarily conserved proteins to meet its own needs in terms of regulating its complex cell cycle and life cycle. To this end, further study of other trypanosome cyclins is likely to yield insights into how this single cell eukaryote evolved a cell cycle with so many unique features.

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The *Trypanosoma brucei* Cyclin, CYC2, Is Required for Cell Cycle Progression through G1 Phase and for Maintenance of Procyclic Form Cell Morphology

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