A PHO80-like Cyclin and a B-type Cyclin Control the Cell Cycle of the Procyclic Form of Trypanosoma brucei*

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Cyclins bind and activate cyclin-dependent kinases that regulate cell cycle progression in eukaryocytes. Cell cycle control in Trypanosoma brucei was analyzed in the present study. Genes encoding four PHO80 cyclin homologues and three B-type cyclin homologues but no GI cyclin homologues were identified in this organism. Through knocking down expression of the seven cyclin genes with the RNA interference technique in the procyclic form of T. brucei, we demonstrated that one PHO80 homologue (CycE1/CYC2) and a B-type cyclin homologue (CycB2) are the essential cyclins regulating G1/S and G2/M transitions, respectively. This lack of overlapping cyclin function differs significantly from that observed in the other eukaryotes. Also, PHO80 cyclin is known for its involvement only in phosphate signaling in yeast with no known function in cell cycle control. Both observations suggest the presence of simple and novel cell cycle regulators in trypanosomes. T. brucei cells deficient in CycE1/CYC2 displayed a long slender morphology, whereas those lacking CycB2 assumed a fat stumpy form. These cells apparently still can undergo cytokinesis generating small numbers of anucleated daughter cells, each containing a single kinetoplast known as a zoid. Two different types of zoids were identified, the slender zoid derived from reduced CycE1/CYC2 expression and the stumpy zoid from CycB2 deficiency. This observation indicates an uncoupling between the kinetoplast and the nuclear cycle, resulting in cell division driven by kinetoplast segregation with neither a priori S phase nor mitosis in the trypanosome.

The eukaryotic cell cycle is governed by multiple regulatory proteins, such as the cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. By the well controlled periodic synthesis and destruction of the cyclins, the corresponding CDK activities go through a corresponding sequential activation and inactivation, which provide the primary means of cell cycle control (1).

The cyclins first were identified as the proteins for which levels oscillate during the progression of the eukaryotic cell cycle. In the budding yeast Saccharomyces cerevisiae, three cyclins (CLN1–CLN3) were found to function in controlling the G1 phase, whereas six closely related B-type cyclins (CLB1–CLB6) were identified in driving the cell cycle from S phase to mitosis (2). These cyclins perform apparently redundant functions because it requires a deletion of all three G1 cyclins to arrest the yeast cells at G1 (3) and a deletion of CLB2 coupled with a deletion of CLB1, CLB3, or CLB4 for an arrest at G2/M (4). These cyclins all bind to the same CDK, CDC28, in a sequential manner to activate CDC28 for regulating yeast cell cycle progression (2). Two other cyclins, PCL1 and PCL2 (5, 6), associate with another CDK, PHO85, for which the primary function is to transduce the phosphate starvation signal in yeast (7), and also are involved in the yeast G1/S transition. However, cyclin PHO80, which binds to PHO85 in transducing the phosphate starvation pathway, apparently is not involved in cell cycle regulation (7). In mammals, at least 16 cyclin-like proteins have been identified, although only 9 of them were found to associate with different CDKs in cell cycle control (1). The cyclin proteins are significantly divergent in structure, but they all share a common region known as the cyclin box domain, which binds and activates the CDKs.

Trypanosoma brucei is a parasitic protozoan and a causative agent of sleeping sickness in Africa. It also generally is regarded as a deeply branched and relatively primitive eukaryote further removed from mammals than yeast. The trypanosome cell division cycle has the usual sequential G1, S, G2, and M phases, but there is a periodic S phase for the unit mitochondrial genome, the kinetoplast, prior to the nuclear S phase, suggesting a well coordinated replication and segregation between the nucleus and the kinetoplast (8). However, treatment of trypanosome cells with the nuclear DNA synthesis inhibitor aphidicolin or the antimicrotubule agent rhizoxin resulted in blocking nuclear DNA synthesis or mitosis without an inhibitory effect on cytokinesis or kinetoplast segregation (9). There is apparently little connection between the nucleus and the kinetoplast in their propagation, which makes the regulation of the trypanosome cell cycle an interesting subject to pursue.

Little is known about how the trypanosome cell cycle is regulated at the molecular level. Two cyclin-like genes, CYC2 and CYC3, were identified recently in T. brucei by rescuing a yeast G1 cyclin deletion mutant (10), and three cdc2-like protein kinase genes also have been cloned from T. brucei (11). However, their roles in cell cycle control have not been determined. CYC2 is a homologue of PHO80 (10). Attempts to disrupt the second allele of the CYC2 gene in T. brucei were unsuccessful, which led to the conclusion that the CYC2 gene is essential for T. brucei viability with little additional information available (10). The trypanosome genome sequence data base, however, has provided us the opportunity to identify...
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MATERIALS AND METHODS

Cell Culture—The procyclic form of T. brucei strain 29–13 (12) was cultivated at 26 °C in Cunningham’s medium supplemented with 10% fetal bovine serum (Atlanta Biological). G418 (15 μg/ml) and hygromycin B (50 μg/ml) were added to the culture medium to maintain the T7 DNA polymerase and tetracycline repressor gene constructs within the cells.

Identification of Cyclin-like cDNAs from T. brucei—Partial genomic sequences of the cyclin-like genes were identified from The Institute for Genomic Research (TIGR) trypanosome genome project sequence data base by the BLAST search program using yeast CLN1–CLN3 and CLB1–CLB8 genes and T. brucei CYC2 and CYC3 genes as the queries. Gene-specific primers then were designed according to the partial sequence information (available upon request) and employed in reverse transcription-PCR (RT-PCR) for rapid amplification of cDNA ends (RACE). First-strand cDNAs were generated from the total RNA of T. brucei with an oligo(dT)15, adaptor primer and Moloney murine leukemia virus reverse transcriptase. The 5′-sequences, resulting in successful cloning of the full-length cyclin-like genes. CYC2 and three of the five newly found cyclin homologues contain the cyclin box domain homologous to that of yeast PHO80 cyclin. We renamed CYC2 as CycE1 (CycE1/CYC2) in our present study and named the other three new cyclins CycE2, CycE3, and CycE4, respectively. CYC3 and the two newly identified cyclin homologues bear sequences very similar to those of the B-type cyclins from a variety of other organisms. Thus, we propose to rename CYC3 as CycE1 (CycE1/CYC3) and to name the other two cyclins CycE2 and CycE3.

Alignment of the conserved cyclin box domains of the cyclins from trypanosomes and those of other eukaryotes showed that trypanosome CycE1/CYC2, CycE2, CycE3, and CycE4 exhibit significant sequence homology to the PHO80 cyclin of S. cerevisiae (Fig. 1A). CycE1/CYC2 bears a 34% sequence identity with PHO80 in the cyclin box domain. The yeast PHO80 cyclin mainly is involved in the phosphate signaling pathway through its association with another CDK, PHO85, and has known roles in cell cycle control (7). Another yeast cyclin subfamily, the PCL cyclins, is known to associate also with PHO85 (7), and the PCL1–PHO85 and PCL2–PHO85 complexes have been shown to play some roles in controlling the G1/S transition in yeast in addition to the CLN1–CLN3 and CDC28 complexes (5, 6). However, the cyclin box domain of trypanosome CycE1–CycE4 exhibits only a very low level of sequence identity with the cyclin box domain of the PCLs (16% between CycE1 and PCL1). Thus it is not possible to determine from the cyclin box domain of trypanosome CycE1–CycE4 whether they function as PHO80 or G1 cyclins.

Trypanosome CycE2 showed, surprisingly, significant sequence homology (50% identity) to the B-type cyclins from plant species such as Glycine max (cyclin cyc5Gm) (17), Catharanthus roseus (cyclin CYM) (18), and Antirrhinum majus (cyclin 1) (Fig. 1B) (19) as well as many B-type cyclin homologues from Arabidopsis thaliana and rice (data not shown). CycE2 exhibited a lower (31–39%) sequence identity to the cyclin box domains of yeast B-type cyclins CLB1–CLB6. Trypanosome CycB3 exhibited, however, a very low level of sequence identity with plant B-cyclins but a moderate level of sequence identity (25%) and similarity (45%) to the B-type cyclins from a variety of other organisms such as Schizosaccharomyces pombe (cyclin CDC13) (20), Drosophila (cyclin CycB) (21), and A. thaliana (cyclin CYC1) (22). The three trypanosomal putative B-type cyclins CycB1/CYC3, CycB2, and CycB3 exhibited a low level of sequence homology (20% identity) to one another, suggesting that they may have originated from different subfamilies. This is in sharp contrast to CycE1–CycE4, which bear

potential protein components of the cell cycle regulatory complex, and the recently discovered RNA interference technique has allowed us to knock down individually the expression of these components in T. brucei to assess from the phenotypes of the knock-down cells their potential functions.

In the present study, we identified from the T. brucei genome data base five new cyclin-like genes other than CYC2 and CYC3 and cloned the full-length cDNAs of four of them. RNAi experiments on the procyclic form of T. brucei showed that a single PHO80 homologue, CycE1/CYC2, played an essential role in the G1/S transition and that a single B-type cyclin homologue CycB2 was indispensable for passing the G1/M boundary (see below). Two different types of anucleated zoid cells, the slender zoid and the stumpy zoid, each containing a single kinetoplast, were derived from these arrested cells; a slender form was produced from a G1/S block, and a stumpy form resulted from a G2/M inhibition. These findings point to not only a simple and novel mechanism of cell cycle regulation but also the occurrence of cell division driven by kinetoplast segregation and cytokinesis in the absence of either nuclear DNA replication or mitosis in T. brucei.
RNA Interference of Expression of Individual Cyclin Genes in the Procyclic Form of T. brucei—We employed the RNAi technique to selectively knock down expression of the seven individual cyclin genes in the procyclic form of T. brucei so that we could explore the potential roles that they each may play during the trypanosome cell cycle. A 300–500-bp DNA fragment of a unique sequence from the coding region of each cyclin gene was amplified by PCR and subcloned into the RNAi vector pZJM (14). The resulting construct then was introduced by electroporation into the procyclic form cells of T. brucei strain 29-13, expressing both tetracycline repressor and T7 RNA polymerase. The stable transfectants were selected under phleomycin and cloned by limiting dilution. Transcription of the DNA insert into double-stranded RNA was induced by adding tetracycline to the culture medium to switch on the T7 promoters. Double-stranded RNA thus synthesized is known to lead to specific degradations of its corresponding mRNA in T. brucei (14–16, 23–26).

The effect of RNAi on individual cyclin gene expression was examined first by semiquantitative RT-PCR analysis. The results, shown in the insets of Fig. 2, indicate that after initiating RNAi for 2 days, the levels of mRNA encoding individual cyclins decreased significantly and became undetectable by RT-PCR. This effective blockade of gene expression also turned out to be highly specific as the levels of the other six cyclin mRNAs were unaffected in each case (data not shown). Effects of individual mRNA depletion on trypanosome replication then were monitored by daily counting of the cultured transfected cells with or without tetracycline induction (Fig. 2). The results, presented as time courses of cell growth, indicated that among the four PHO80 homologue-deficient cell lines, only the growth of CycE1/CYC2-deficient cells was inhibited significantly (Fig. 2A). The growth of cells deficient in CycE2 or CycE4 assumes the same rate as the wild type cells, whereas the CycE3-deficient cells grow with an ~50% reduced rate.
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Cell Cycle Defects Identified in the Cyclin-deficient Trypanosome Cells—To ascertain the specific point of cell cycle arrest among the individual cyclin-deficient trypanosome cell lines, we stained the cells with propidium iodide and performed FACScan to separate them by their DNA content. The results, which are presented in Fig. 3, show that among the CycE1/CYC2-deficient cells, those in the G1 phase are increased to 90% of the total population, whereas the G2/M phase cells are decreased to make up 10% of the total population. The S phase cells are essentially undetectable after 9 days of induced RNAi. These data suggest that the transition from G1 to S phase is inhibited significantly. CycE1/CYC2-deficient cells showed a slight (10%) increase in G2/M phase cells with a corresponding decrease in S phase cells without an appreciable change in the G1 population over a period of 9 days. This observation suggests that the transition from G1 to S phase is slowed down slightly without arresting the progression of the cell cycle. CycE3 thus still may perform a role, albeit nonessential, in the G1/S transition. Consistent with their lack of effect on cell proliferation, a deficiency of CycE2 or CycE4 in trypanosome cells did not result in any apparent change in the profiles of the cells compared with wild type cells (data not shown). These two proteins probably are not involved in cell cycle regulation. We thus conclude that CycE1/CYC2 is the dominant as well as the essential G1 cyclin, whereas CycE3 plays an auxiliary role in the G1/S transition of T. brucei in procyclic form.

Depletion of CycE2 resulted in the arrest of almost 90% of the cell population in the G2/M phase within a relatively short period of 24 h (Fig. 3C). This suggests that CycE2 is a highly essential mitotic cyclin in the procyclic form of T. brucei, mediating the progression through G2 to M phase. The CycB3-deficient cells showed a slight (−10%) increase in the number of G2/M phase cells with a corresponding decrease in the number of G1 phase cells after 9 days (Fig. 3D), suggesting an auxiliary role of CycB3 in promoting the cells through the G2/M boundary. Finally, depletion of CycB1/CYC3 had no detectable effect on the profile of cells in FACScan (data not shown), suggesting that CycB1/CYC3 likely is uninvolved in cell cycle control. The results derived from these experiments thus indicate once again only one essential/dominant mitotic cyclin, CycB2, and one auxiliary mitotic cyclin, CycB3, in the procyclic form of T. brucei. This scheme of cyclin-regulated G1/S and G2/M transition is perhaps the simplest among all those known to date.

Cellular Morphology of the Cyclin-deficient T. brucei Procyclic Form Cells—Microscopic examination of the CycE1/CycC2-deficient cells indicated an obvious morphological change from...
the original wild type. A long slender morphology of the cells became obvious after induction of RNAi for 3 days, becoming even longer and more slender after 9 days (Fig. 4A, middle panel). It was apparently the consequence of a blocked G1/S transition. Most of the population (80–90%) contained one nucleus and one kinetoplast (1N1K) in each cell as in the wild type cells. But, unlike the wild type cells, about 7% of the population (Fig. 4B, middle panel), appearing shorter and smaller in size, turned out to contain no nucleus but one kinetoplast in each cell upon staining with 4',6-diamidino-2-phenylindole (DAPI) and examination under a fluorescence microscope. A, left-hand panel, the control cells harvested after 9 days demonstrated the presence of all three types of cells (one nucleus and one kinetoplast (1N1K), one nucleus and two kinetoplasts (1N2K), and two nuclei and two kinetoplasts (2N2K)). Middle panel, the CycE1/CYC2-deficient cells 9 days after RNAi induction had the 1N1K phenotype but became unusually slender with an average length twice as long as the control cells. Some of the slender cells were shorter in length but contained only one kinetoplast without a nucleus. They were named the “slender zoids.” Right-hand panel, the CycB2-deficient cells harvested 2 days after RNAi induction each contained a significantly enlarged nucleus with only one kinetoplast. Some cells of the same size and with an equally fat and stumpy shape contained no nucleus and were designated the “stumpy zoids.” Size bars, 3 μm. B, quantification of the different cell types in the three cell samples. Individual cells were scored under a fluorescence microscope for the number of kinetoplasts (K) and nuclei (N). Data are presented as the mean percent ± S.E. of total cells counted (>200) from three independent experiments.

After 24 h of induced RNAi, the CycB2-deficient cells became, however, fat and stumpy compared with the control cells, which had not undergone RNAi induction (Fig. 4A, left panel). The majority of the cells (70–80%) contained one kinetoplast and one significantly enlarged nucleus (Fig. 4A, right panel), suggesting that DNA replication occurred but that mitosis failed, leading to the arrest of cell growth. About 13% of the population also turned out to be zoids (Fig. 4B, right panel). However, in contrast to the slender phenotype of zoids observed among the CycE1/CYC2-deficient cells, the CycB2-deficient zoids seemed fat and stumpy, similar to the CycB2-deficient cells that contained both the kinetoplast and the enlarged nucleus (Fig. 4A, right panel). These stumpy zoids, which are apparently the daughters of cells blocked at the stage of mitosis, provided a clear indication that cell division of the procyclic form of T. brucei can occur without a priori nuclear mitotic division. This abortive cell division, which is driven most likely by the segregation of the kinetoplast, provides an intriguing example of cell division without nuclear mitosis.

DISCUSSION
In the present investigation, we performed molecular identification and functional analysis of seven cyclin homologues in the procyclic form of T. brucei as a first step to dissect the molecular mechanism of cell cycle control in this primitive eukaryote. One of the yeast PHO80 cyclin homologues, CycE1/CYC2, turned out to be an essential G1 cyclin, whereas a close homologue of plant B-type cyclin CycB2 was identified as an essential mitotic cyclin in trypanosomes. Although the complete spectrum of cyclin involvement in regulating the trypanosome cell cycle is not yet available, the apparent simple scheme

![Image](https://example.com/image.png)
reflected by the indispensability of only two individual cyclins suggests that T. brucei has a very primitive molecular mechanism in cell cycle regulation.

The first intriguing observation from our present study is that yeast PHO80, the closest homologue of trypanosome CdcE1/CycC2, is a cyclin present at a constant level in yeast and is not involved in cell cycle regulation (7). It binds and activates a CDK, PHO85, in yeast in responding to phosphate starvation (7). PHO85 has another subfamily of cyclin partners, the PCLs, some of which (PCL1, PCL2, etc.) have a pattern of cell cycle-regulated expression (5, 6, 31, 32). Although deletion of PCL1 or PCL2 did not lead to any detectable phenotype, \( \Delta \text{cln2} \Delta \text{cln1} \text{pcl1} \) and \( \Delta \text{cln1} \Delta \text{cln2} \text{pcl2} \) caused arrest of yeast cells in the G1 phase, thus suggesting some roles for PCL1 and PCL2 in controlling the G1/S transition (5, 6). CycE1/CycC2 shares, however, only a very limited sequence identity with PCL1 (16%) and PCL2 (18%). This trypanosome cyclin was found previously to be associated with a cdc2-related kinase (CRK) in T. brucei by Van Hellemond et al. (10) using immunoprecipitation and a yeast two-hybrid system. CRK3 shares 44% sequence identity with PHO85 and 48% identity with CDC28. It could perform the primary function of PHO85 and/or CDC28 in regulating the G1/S transition in trypanosomes with its kinase activity regulated primarily by a PHO80-like cyclin, CycE1/CycC2.

The cyclin box domain of CycB2, the essential mitotic cyclin in trypanosomes, has significant sequence identity with the cyclin box domains of the plant B-type cyclins (Fig. 1B). It is not known whether these plant B-type cyclins also play a dominant role in controlling G2/M transition in the respective plants, but the B-type cyclins in budding yeast are well known for their functional redundancy. Among CLB1, -2, -3, and -4, multiple deletions, which always include the deletion of CLB2, are required to arrest the yeast cell cycle (4). CLB2 shows the highest sequence similarity to the S. pombe B-cyclin homologue CDC13 (20), which bears significant sequence homology with CycB3 from T. brucei (Fig. 1C). However, the latter plays only an auxiliary role in regulating the trypanosome cell cycle through the G2/M transition. It thus has become a futile effort to try to compare the structures and functions of cyclins from different species to gain a more in-depth understanding of the cyclins newly identified in T. brucei. The cyclins may have evolved fairly rapidly in response to changes in a complex extracellular environment to maintain a robust cell cycle progression and metabolism of nutrients (7). The living environment for trypanosomes oscillating between mammalian bloodstreams and tsetse midguts can be relatively stable. A more conserved, and thus more simplistic, primitive, and ancient cyclin system thus still could be operating well in this organism ready to be explored as a model.

Another interesting observation resulting from our present study is the identification of two types of zoids from cell cycle arrest at two different checkpoints. The slender zoid produced from a blocked G1/S transition suggests the occurrence of cell division without an S phase, whereas the appearance of the stumpy zoid under an inhibited G2/M transition indicates cell division without mitosis. Thus trypanosome cells are capable of dividing without progressing through their regular cell cycle. This finding is in agreement with that of Ploubidou et al. (9), who indicated that the nucleus and the kinetoplast in T. brucei each have a distinct S phase followed by a segregation period prior to cell division. The segregation of nuclear DNA depends on the spindle, whereas the flagellum basal bodies are instrumental for kinetoplast DNA separation. T. brucei cytokinesis is apparently not dependent upon either nuclear DNA synthesis or mitosis. Thus among some aphidicolin-treated trypanosome cells, Ploubidou et al. (9) observed basal body segregation, which is apparently capable of dividing the cell into two daughter cells with one of the two forming the zoid. When the expression of an essential G1 cyclin CycE1/CycC2 was knocked down in our present study, kinetoplast segregation and cytokinesis proceeded apparently normally, leading to successful cell division to generate the zoids. However, because of the blocked S phase in these cells, daughter cells either with or without a nucleus assumed a slender appearance (Fig. 4A, middle panel). Conversely, in the absence of an essential mitotic cyclin, CycB2, the cells were blocked from mitosis; nonetheless, cytokinesis proceeded unhindered, and the kinetoplast went through its segregation resulting in cell division. Because the cells already had gone through a normal S phase, daughter cells with or without a nucleus appeared to have an equally full size with a stumpy shape (Fig. 4A, right panel).

This observation leads to an interesting elucidation that trypanosome cell division can be driven by the synthesis and segregation of the kinetoplast alone while the cell cycle is arrested. But, because the dyskinetoplastic bloodstream form of trypanosomes are known to divide in a normal manner (33, 34), cell division apparently can occur after mitosis in the absence of the kinetoplast. There must be thus two separate schemes capable of driving the division of trypanosome cells. They are apparently independent of each other, and yet they are well coordinated in the wild type cells resulting in cell divisions in a synchronous manner. The molecular mechanisms underlying two different pathways for cell division and the coordination between them may constitute one of the most interesting aspects of trypanosome cell biology.

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