Isolation of Trypanosoma brucei CYC2 and CYC3 Cyclin Genes by Rescue of a Yeast G₁ Cyclin Mutant

FUNCTIONAL CHARACTERIZATION OF CYC2*

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Jaap J. Van Hellemond§§, Philippe Neuville$$, Ralph T. Schwarz§, Keith R. Matthews, and Jeremy C. Mottram***

From the ¶Wellcome Centre for Molecular Parasitology, University of Glasgow, Anderson College, Glasgow G11 6NU, Scotland, United Kingdom, #Zentrum fur Hygiene und Medizinische Mikrobiologie, Philips-Universitat, 35037 Marburg, Germany, and |School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Two Trypanosoma brucei cyclin genes, CYC2 and CYC3, have been isolated by rescue of the Saccharomyces cerevisiae mutant DL1, which is deficient in CLN G₁ cyclin function. CYC2 encodes a 24-kDa protein that has sequence identity to the Neurospora crassa PREG1 and the S. cerevisiae PHO80 cyclin. CYC3 has the most sequence identity to mitotic B-type cyclins from a variety of organisms. Both CYC2 and CYC3 are single-copy genes and expressed in all life cycle stages of the parasite. To determine if CYC2 is found in a complex with previously identified trypanosome cdc2-related kinases (CRKs), the CYC2 gene was fused to the TY epitope tag, integrated into the trypanosome genome, and expressed under inducible control. CYC2ty was found to associate with an active trypanosome CRK complex since CYC2ty bound to leishmanial p12k, and histone H1 kinase activity was detected in CYC2ty immune-precipitated fractions. Gene knockout experiments provide evidence that CYC2 is an essential gene, and co-immune precipitations together with a two-hybrid interaction assay demonstrated that CYC2 interacts with CRK3. The CRK3-CYC2ty complex, the first cyclin-dependent kinase complex identified in trypanosomes, was localized by immune fluorescence to the cytoplasm throughout the cell cycle.

The parasitic protozoon Trypanosoma brucei belongs to the family of Trypanosomatidae, which is thought to be one of the earliest branching eukaryotic families to contain a mitochondrion (1). The African trypanosome, T. brucei, is responsible for the clinically important diseases, sleeping sickness in humans and nagana in cattle. Trypanosomes have a complex life cycle that alternates between insect and mammalian hosts with different developmental forms having specific roles within each host. The rapidly dividing forms such as the long slender bloodstream trypomastigote and the tsetse fly vector establish an infection in the host. The parasite also has a nondividing stumpy bloodstream form, which is arrested in the G₀/G₁ phase of the cell cycle and is reprogrammed for survival when the organism passes into the tsetse (2). During the trypanosome life cycle there is an integral link between the control of the cell cycle and parasite differentiation (3), and there is a special need to coordinate replication and segregation of its single organelles: the nucleus, mitochondrion, and flagellum (4). Thus, trypanosomes have added complexity in cell cycle control not present in many single-cell eukaryotes such as yeast. The similarities and differences between the regulation of the cell cycle in trypanosomatids and other eukaryotes provide important information about the evolution of regulatory systems controlling the cell cycle. However, little is known in molecular terms of how the trypanosome cell cycle is controlled.

Cell cycle progression in eukaryotes is regulated by a family of cyclin-dependent kinases (CDKs) that require association with cyclin regulatory partners for activity. The first CDKs identified, Schizosaccharomyces pombe cdc2 and Saccharomyces cerevisiae CDC28, are highly conserved in evolution and were the first of the CDK family (CDK1) to be shown to have a central role in cell cycle control (5, 6). CDKs have been shown to have roles in other cellular functions such as transcriptional regulation and response to stress (7). The activity of CDKs can be controlled post-translationally by a number of different mechanisms: the association of the kinase subunit with its positive regulatory cyclin partner, phosphorylation of conserved sites, which can stimulate or inhibit kinase activity, and the binding of CDK inhibitor proteins (8). Since binding of a cyclin partner is essential for full CDK activity, the concentrations of the cyclins are tightly controlled by transcriptional regulation as well as a timing of proteolysis (9). In addition to the concentration of the cyclin “cycling” during the cell cycle, cyclins are characterized by the presence of a 100-amino acid region of sequence similarity to the consens cyclin box, which is involved in binding to their CDK partner (10, 11). So far, a large family of cyclin genes has been identified in many species, of which the majority are involved in regulation of the cell cycle (8). Mitotic cyclins contain a characteristic nine-amino acid destruction box motif, which targets cyclins for degradation at the end of mitosis (12). G₁ cyclins are more divergent, and some have C-terminal PEST sequences, which are involved in their degradation (13). The budding yeast CDKs, CDC28 and

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** Contributed equally to this study.

† Medical Research Council Senior Fellow and to whom correspondence should be addressed: Wellcome Centre for Molecular Parasitology, University of Glasgow, The Anderson College, 56 Dumbarton Rd., Glasgow G11 6NU, Scotland, UK, Tel.: 44 141 330 3745; Fax: 44 141 330 5422; E-mail: j.mottram@udcf.gla.ac.uk

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1 The abbreviations used are: CDK, cyclin-dependent kinase; CRK, cdc2-related kinase; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); PARP, poly(ADP-ribose) polymerase; PEST, proline-lysine glutamic acid serine-threonine.
PHO85, bind at least three different classes of cyclin, each containing between two and five members, giving a high level of functional redundancy (14). Higher eukaryotes, on the other hand, have a multiplicity of CDKs (nine at the last count) and cyclins (A-T) (8).

A complex regulation of the cell cycle in trypanosomatids is supported by the identification of a large family of genes with homology to CDKs. These genes were termed cdc2-related kinase (CRK) genes, as no cyclin partners were known to bind these kinases when they were first described. CRKs have been identified in T. brucei (15, 16), Leishmania (17–19), and Crithidia (20). The trypanosomatid CRKs have several features in common with yeast and human CDKs (16, 18, 21), including 50–55% amino acid sequence identity with CDK1 from a range of organisms (15), a recognizable (but divergent) “PSTAIR” box, which is a domain shown to be important for cyclin binding (11), and key threonine and tyrosine residues known to be important sites of phosphorylation in yeast and mammalian (9). The CRKs also have sequence features that distinguish them from yeast or mammalian CDKs, including an N-terminal extension for CRK2 and CRK3 (15) and two additional domains within the catalytic domain of the kinase for CRK4 (20).2 Hence the regulation of the trypanosome cell cycle, whose cell biology differs significantly from many other eukaryotic organisms, may also differ at the molecular level.

In contrast to the CRKs, much less is known about the activating cyclin partners in trypanosomes. So far only one putative cyclin gene, CYC1, has been identified (22). Despite evidence to suggest that this gene encodes a mitotic cyclin (22), other studies provide no evidence that CYC1 is a functional cyclin.3 CYC1 may be involved, however, in regulating differentiation of the bloodstream form into the procyclic form (23). Identification of cyclin genes has proven to be difficult due to their divergence at the primary amino acid level (24). Hence, it is crucial to define cyclins on a functional basis to validate their identification. In this study we used a functional complementation assay to isolate trypanosome cyclins based on their ability to rescue a S. cerevisiae G1 cyclin conditional mutant. A variety of heterologous cyclins from species as divergent as Homo sapiens (25), Drosophila (26), and Arabidopsis (27) have been shown to rescue cln1,2,3 lethality. This functional complementation assay enabled us to identify two novel T. brucei cyclin genes, CYC2 and CYC3, and to define the first trypanosome cyclin-dependent kinase complex.

**EXPERIMENTAL PROCEDURES**

Recombinant DNA Techniques—All standard DNA techniques were performed as described by Sambrook et al. (28). Yeast transformation was performed according to the lithium acetate procedure (29). Plasmid recovery from yeast used the glass bead-phenol technique (30) followed by transformation of Escherichia coli. Plasmid isolation from E. coli was performed using Qiaprep columns (Qiagen). DNA sequencing was performed using a ABI automatic sequencer.

**Complementation of a Yeast Cyclin Mutant—**The complementation study was performed with the DL1 strain of S. cerevisiae in which the three CLN genes (CLN1, CLN2, and CLN3) were inactivated, and a chimeric gene consisting of the GAL1 promoter fused to the CLN2 protein-coding region was integrated at the LEU2 chromosomal locus (25). The GAL1 promoter directs high level expression when the cells are grown on galactose-containing medium but is repressed if the cells are grown on a medium containing glucose. DL1 cells are able to grow on galactose-based medium but arrest in G1 on a glucose-based medium. DL1 cells were transformed with a T. brucei cDNA library, which was constructed in the yeast expression vector pRS416-met (31). The pRS416 vector carries the URA3 gene as a selectable marker, and the cDNAs are fused with the MET25 promoter at the 5′ end and the CYC1 terminator at the 3′ end.

**Antiserum—**Antipeptide antisera were raised against the last 9 amino acids of CRK1 (EHPPYSVFEF) and the last 16 amino acids of CRK2 (EVREEVEKLMRFNGA). Peptides were conjugated to keyhole limpet hemocyanin and used to immunize rabbits using standard procedures (32). Antiserum specific to CRK3 was raised by immunization of rabbits with purified recombinant CRK3 that was tagged with six histidines at its N terminus (33). The mouse monoclonal antibody BB2 (34) was used as the anti-TY antibody.

**Creation of TY-tagged CYC2 (CYC2ty)—**Two annealed oligonucleotides (OL49 and OL50, see Table I), which composed the TY-epitope flanked by BglII-compatible overlaps, were ligated into the unique BglII site near the C terminus of CYC2 (Fig. 2), after which proper integration of the TY tag was confirmed by sequence analysis. Subsequently, CYC2ty was cloned in the unique EcoRI and XhoI sites of the yeast expression vector pRS416-met (31) to produce plasmid pGL387. In addition, CYC2ty was also cloned into the single BglII site of the tetracycline-inducible trypanosome expression vector pHD675 (35) to produce plasmid pGL289.

**Transfection of T. brucei and Expression of CYC2ty—**Procytic form T. brucei EATRO 795 (36) or T. brucei STIB 247 (37) were grown at 28 °C in SDM-79 medium (38) containing 10% (v/v) heat-inactivated fetal bovine serum. Transfection of procytic trypanosome cells was performed by an electroporation method. 107 trypanosome trypanosomes from a mid-log phase culture were harvested and washed in transfection buffer (132 mM NaCl, 8 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 1.5 mM MgCl2·6H2O, and 90 μM Na2CO3·H2O), after which the cells were resuspended in 500 μl of transfection buffer with 5–10 μg of DNA and electroporated (1500 volts/25 microfarads). Subsequently, cells were incubated in SDM-79 medium at 28 °C for 16 h, after which antibiotics were added, and cells were cloned directly by limiting dilution.

Procytic EATRO 795 cells were transfected with the pHD449 vector for constitutive expression of the tetracycline-repressor protein (35). Subsequently, a clonal population of these cells were transfected with the CYC2ty expression vector (pGL289, linearized with NotI), after which cells were re-cloned by limiting dilution. Induction of expression of CYC2ty was initiated in mid-log phase cultures by the addition of tetracycline (50 μg/ml), and the cells were harvested 16 h after induction by centrifugation and subsequent washing with phosphate-buffered saline (18).

**Immunoblotting—**Cell pellets or protein samples were resuspended in Laemmli buffer, separated on 10 or 12.5% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Western blots were performed as described before (21) with a 1:100 dilution of the crude antisem directed to either CRK1, CRK2, CRK3 or with a 1:200 dilution. After which they were re-cloned by limiting dilution. Induction of expression of CYC2ty was initiated in mid-log phase cultures by the addition of tetracycline (50 μg/ml), and the cells were harvested 16 h after induction by centrifugation and subsequent washing with phosphate-buffered saline (18).

**Primer sequences**

| Primer name | Sequence |
|-------------|----------|
| CYC2KO5'5′ | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| CYC2KO3'5′ | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| CYC2KO3'3′ | 5′-GAATTCCTGGTGCTTTCATTTCC-3′ |
| OL11 | 5′-GCCATCGTGTCGCCAACCTTC-3′ |
| OL15 | 5′-CCGTTGGGCTGTGACCTGGCAAAAGG-3′ |
| OL40 | 5′-GATCTGACGTCAGGCTGCATTAACACAGGATTCCATGCACA-3′ |
| OL50 | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| OL243 | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| OL434 | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| OL446 | 5′-GATCTGCGTGCAAGCGCGGCCAAGAGGC-3′ |
| OL543 | 5′-GAATTCCTGGTGCTTTCATTTCC-3′ |

**TABLE I**

2 J. R. Ford and J. C. Mottram, unpublished information.
3 T. C. Hammarton, J. R. Ford, and J. C. Mottram, manuscript in preparation.
bated for 2 h with 40 μl of either control or p12mut or p13mut beads (18), after which the beads were extensively washed with lysis buffer and used for immunoblotting or histone H1 kinase assays.

Immunoprecipitations and Histone H1 Kinase Assays—Immunoprecipitations and kinase assays were performed as described previously. S. 100-cell extracts, prepared as described above, were incubated with 50 μl of serum in the absence or presence of 5 μg ml⁻¹ peptide for 60 min at 4 °C. Subsequently, 25 μl of protein A-Sepharose (Amersham Pharmacia Biotech) was added and incubated for a further 30 min at 4 °C. After extensive washing of the Sepharose beads with lysis buffer, the beads were either resuspended in Laemmli buffer and used for SDS-polyacrylamide gel electrophoresis and immunoblotting or resuspended in kinase assay buffer (50 mM MOPS, pH 7.2, 20 mM MgCl₂, 2 mM dithiothreitol, 10 mM EGTA, 250 μg ml⁻¹ histone H1 (Life Technologies, Inc.) and 4 μM ATP (0.1 μM of [γ⁻³²P]ATP) and incubated for 30 min at 30 °C for a histone H1 kinase assay (21). Kinase activity of SDS-polyacrylamide gel electrophoresis-separated samples was examined by drying of the gel and exposure to an X-ray film.

Two Hybrid Interaction Assay—A yeast two-hybrid interaction assay was performed using the LexA Hybrid Hunter system (Invitrogen). T. brucei CRK1 and CRK3 were cloned into the EcoRI/PstI site of pHybLexZeo by constructing unique EcoRI and PstI sites on the 5′ and 3′ ends of the coding region by PCR. The inserts for CRK1 (pGL176) and CRK3 (pGL177) were sequenced to confirm in-frame fusions with LexA and to check that no PCR mutations had been introduced. The CYC2 and CYC3 genes were cloned into the HindIII/XbaI sites of the pYES2Trp vector using the same PCR-based approach to give plasmids pGL182 and pGL181, respectively. These produced fusions with the B42 domain. Bait and prey plasmids were transferred sequentially into S. cerevisiae strain L40 (Invitrogen), and protein:protein interactions were assessed by β-galactosidase activity using a filter lift assay as described by the manufacturer. Expression of the fusion proteins was checked by Western blot analysis using antibodies (Invitrogen) against LexA and the V5-epitope, which is fused to the B42 domain, according to the manufacturer’s protocol.

Disruption of the CYC2 Gene—In the absence of a sufficient 5′ and 3′ sequence flanking the CYC2 gene, a gene disruption approach was adopted to knock out CYC2 gene function rather than a conventional gene deletion. The CYC2 gene was disrupted by transfection with BLE and PAC resistance knockout constructs. These constructs were designed to replace 160 bp of sequence in the middle of the CYC2 open reading frame with the required antibiotic resistance gene. Correct transcription and post-transcriptional processing of the drug resistance genes relied on 5′ and 3′ PAP control sequences. A 320-bp DNA fragment containing the 5′ end of CYC2 was amplified from plasmid pGL163 with primers CYC2KO5′5′ and CYC2KO5′3′ and cloned into pCR-script. A 320-bp DNA fragment containing the 3′ end of CYC2 was amplified from plasmid pGL163 with primers CYC2KO3′5′ and CYC2KO3′3′ and also cloned into pCR-script. CYC2KO5′5′ and CYC2KO3′5′ were engineered with SphI and XbaI sites, respectively, whereas CYC2KO5′3′ and CYC2KO3′3′ were engineered with XbaI and EcoRI sites, respectively. pBluescript-based plasmid pGL110 containing the 5′-PAP-PAC-3′-PAP cassette (derived from pJF44 (39)) was used to make the PAC-resistant CYC2 deletion constructs. The 320-bp KpnI/EcoRI fragment containing the CYC2 3′ end was cloned into KpnI/EcoRI-digested pGL110. Subsequently, the CYC2 5′ flank was cloned into SphI/XbaI sites to give pGL2. The BLE-resistant cassette (pGL1) was generated by removing the 1.7-kb 5′-PAP-PAC-3′-PAP fragment by digestion with XbaI/KpnI and replacing with the 1.5-kb XbaI/KpnI 5′-PAP-BLE-3′-PAP fragment from pGL108. The disruption cassettes were excised by digestion with SphI and EcoRI, and the insert was gel-purified using a Qiextract kit (Qiagen). Approximately 5 to 10 μg of DNA was used for each transfection with 4 × 10⁵ mid-log procyclic STIB 247 trypanosomes, after which transfected parasites were cloned by limiting dilution.

PCR analysis on integration of the CYC2 disruption cassettes was performed on chromosomal DNA prepared by a mini-prep procedure (40). Distinct PCR experiments were performed in which five oligonucleotides in total were used (Table I, see also Fig. 5A).

Immunofluorescence—Logarithmic phase procyclic form trypanosomes were fixed in 1% paraformaldehyde to express CYC2ty by the addition of 1 μg ml⁻¹ tetracycline. Cells were harvested, prepared, and slides were fixed by immersion in −20 °C methanol for at least 30 min. After fixation, slides were rehydrated in phosphate-buffered saline for 5 min and then incubated with the BB2 monoclonal antibody for 45 min. After washing, anti-mouse fluorescein isothiocyanate conjugate diluted 1:50 was applied to the slides for a further 45 min, and the cells were washed and counterstained with 4,6-diamidino-2-phenylindole (1 μg/ml) and mounted in MOWIOL (Harlow Chemical Co., UK) containing 1 mg ml⁻¹ phenylenediamine as an anti-fading agent. Cells were visualized on a Zeiss Axioscope microscope, and images were captured and processed using a Hamamatsu CCD camera, NIH image 1.58, and Adobe Photoshop 5.

RESULTS

Isolation of T. brucei Cyclins by Complementation of a Yeast CLN Mutant—The low level of sequence identity between cyclins of different species precludes in general the use of PCR-based approaches to identify cyclins. Hence, we used a functional complementation assay to isolate trypanosome cyclins based on their ability to rescue a S. cerevisiae G₁/S phase cyclin conditional mutant. Yeast DLI cells were transformed with a T. brucei cDNA library cloned in the yeast expression vector pRS416-met (31). 5 × 10⁵ cells were plated on solid medium lacking uracil but containing glucose, and 200 transformed colonies were obtained. Of these 157 were plasmid-independent revertants that had lost the plasmid and were able to grow on glucose plates containing uracil and the antimitabolite 5-fluoro-orotic acid. The 43 remaining plasmids were recovered in E. coli and used to transform the DLI yeast strain a second time. Transformants obtained with six of the plasmids were able to grow on the glucose-based medium, showing that these plasmids were able to complement the cyclin deficiency (Fig. 1). The six cDNAs were grouped into two classes by restriction analysis and partial sequencing. The two different cDNAs of 870 bp (pGL163) and 1.79 kb (pGL164) were then sequenced, and the genes were named CYC2 and CYC3.

CYC2 and CYC3 Have Homology to Cyclins—CYC2 is predicted to encode a 24.3-kDa protein (CYC2) of 211 amino acids, with an isoelectric point of 6.82. CYC3 is predicted to encode a 46.5-kDa protein (CYC3) of 414 amino acids, with an isoelectric point of 7.24. Computer searches of data bases with the CYC2 and CYC3 protein sequences found significant homology to a wide range of cyclins. This homology is, however, restricted to the stretch of about a 100 amino acids known as the cyclin box, which is involved in binding of the CDK partner (9). The cyclin box of CYC2 has 58% identity with that of Neurospora crassa PREG1 regulatory protein (41), 25% identity with the cyclin box of S. cerevisiae PHO80 cyclin (42), and 22% identity with the cyclin box of human cyclin D3 (43) (Fig. 2B). The cyclin box of CYC3 has 32% identity with S. cerevisiae CLB1 (44) and 32% identity with CLB2 (45) (Fig. 2C). Apart from the cyclin boxes, the other regions of CYC2 and CYC3 demonstrate no significant homology to any other known protein, although CYC3 does contain a destruction box-like motive (RGTLLVPRN, see
bloodstream and procyclic forms. Although it is a common feature of mRNA molecules from trypanosomes to contain substantial 5'- and 3'-untranslated regions, the mRNA encoding CYC2 is unusually long, as it is more than 5 times the length of the open reading frame. These extensive flanks may have a role in the post-transcriptional control of CYC2 expression, since 3'-untranslated regions in the mRNA of some trypanosomes genes have a function in the control of gene expression (47, 48).

**Induced Expression of CYC2ty**—The sequence encoding the TY epitope (34) was cloned into the CYC2 gene to produce a fusion protein in which the TY tag was located outside the cyclin box and nine amino acids from the C terminus (Fig. 2A). Since constitutive overexpression of cyclin genes might be toxic to the cell, we used the tetracycline-inducible expression system present in the DH10B strain (25) to express CYC2ty (35, 49). A single 27-kDa protein corresponding to CYC2ty could be detected by Western blotting with the anti-TY monoclonal antibody (34) in lysates from a CYC2ty clone grown in the presence of tetracycline (Fig. 4A, lane 1). No CYC2ty could be detected in lysates from the same clone grown in the absence of tetracycline (lane 2), showing that expression of CYC2ty is tightly controlled. The TY antibody was highly specific, as only a single band was detected in CYC2ty-expressing cells (lane 3) that could be competed out by the addition of TY peptide (lane 4).

Since the introduction of the TY tag in CYC2 could have destroyed its in vivo function, we analyzed the ability of CYC2ty to complement the *S. cerevisiae* DL1 (25). CYC2ty was found to complement the DL1 cells to the same extent as the CYC2 gene, whereas the control vector without the insert was unable to complement these cells (Fig. 4B). Therefore, the *T. brucei* CYC2 gene containing the TY epitope retains cyclin function in *S. cerevisiae*.

The trypanosome clones were analyzed for their phenotype upon induction of CYC2ty. Although CYC2ty was abundantly expressed in these cells (Fig. 4A) no aberrant phenotype could be observed. Their morphology and growth did not significantly differ from control cells (not shown). In addition the same result was found with native CYC2. These results demonstrate that overexpression of CYC2 does not interfere with cell growth or division.

**p13wcl and p12bscl Selection**—We recently described the cloning of the *L. mexicana* CKS1, which is a homologue of yeast...
SUC1 (21). Leishmanial p12<sub>c</sub> and yeast p13<sub>sa</sub> proteins, when bound to Sepharose matrix, interact with a cdc2-related kinase from Leishmania, which is encoded by the LmcRK3 gene (18). LmcRK3 has histone H1 kinase activity (18). To determine if a similar histone H1 kinase activity exists in the T. brucei procyclic form, S-100 extracts were prepared and affinity-selected on Leishmanial p12<sub>c</sub> or yeast p13<sub>sa</sub> beads. Kinase activity was assessed by the ability of selected proteins to phosphorylate the exogenous substrate histone H1 (Fig. 5A). A comparable activity was detected in wild type cells (lanes 1–3) or the transgenic line expressing CYC2ty (lanes 4–6), although there appeared to be a weaker interaction with p13<sub>sa</sub> than p12<sub>c</sub>, CYC2ty could only be detected on the fraction derived from CYC2ty-expressing cells that was selected on p12<sub>c</sub> beads (lanes 5), suggesting that CYC2ty interacts with a trypanosome CRK. The lack of binding of CYC2ty to p13<sub>sa</sub> suggests that CYC2ty is not part of the CRK3-containing histone H1 kinase that binds p13<sub>sa</sub>. Histone H1 kinase activity was detected following immunoprecipitation with the anti-TY antibody from procytic cell extracts expressing CYC2ty (Fig. 5C). Specificity of the immunoprecipitations was shown by the significant reduction in kinase activity by preincubation of the antibody with its corresponding peptide (lane 2).

**The Identification of CRK-Cyclin Complexes—**A two hybrid protein:protein interaction assay was performed with the T. brucei CRK1 and CRK3 proteins as bait and the CYC2 and CYC3 proteins as prey. A strong interaction was detected between CRK3 and CYC2 (Fig. 6A). No activation was observed for CRK3 or CYC2 fusion proteins in combination with control plasmids lacking an insert (not shown), demonstrating that the interaction is specific for the presence of both the CYC2 and the CRK3 fusion proteins. No CYC2-CRK1, CYC3-CRK1, or CYC3-CRK3 interactions were detected, although Western blots with antibodies against the LexA domain or the V5 epitope present in the BD42 domain confirmed that these fusion proteins were expressed in all combinations (not shown). Interaction of CYC2 with known T. brucei CDKs was also investigated by co-immune precipitations on S-100 lysates of CYC2ty-expressing cells. Immune-precipitations were carried out with antibodies directed against CRK1, CRK2, and CRK3, which were subsequently prepared for Western blot analysis using the anti-TY
antibody. As shown in Fig. 6B, CYC2ty could be detected in the fraction that was precipitated with the antibody directed against CRK3 (lane 5), whereas no CYC2ty could be detected in the fractions that were precipitated with antibodies directed against CRK1 and CRK2 (lanes 2 and 4). These two sets of corroboration results demonstrate that CYC2 binds to CRK3 in T. brucei and, therefore, demonstrate that a CDK complex comprising a cyclin-dependent kinase and a cyclin subunit functions in trypanosomes.

Localization of CYC2ty—The subcellular location of CYC2ty in procyclic trypanosomes was investigated by immunofluorescence with anti-TY antibody (Fig. 7). A clear fluorescent signal was detected in all parts of the cell body (lower panel). This fluorescence was specific, since significant fluorescence was not detected in wild type cells lacking CYC2ty or in CYC2ty-expressing cells that were incubated without primary antibody (not shown). Notably, although CYC2ty was localized in all parts of the complete cell body (lower panel), it was not localized in the nucleus, since the fluorescence signal of the TY-staining did not co-localize with DNA staining with 4,6-diamidino-2-phenylindole (upper panel). Although there was found to be variation in signal intensity within the population of trypanosomes (lower panel), the variation did not correlate with the position of the trypanosome in the cell cycle. A second procyclic cell line expressing CYC2 with an N-terminal TY tag was used to investigate the position of the TY tag does not influence the subcellular location of the tagged CYC2. Therefore, the immunofluorescence data strongly suggest that CYC2, when overexpressed in the trypanosome, is localized in the cytoplasm and not in the nucleus, and no cell cycle stage-specific staining could be detected with these overexpressing cell lines.

CYC2 Gene Disruption—Since CYC2 is a single copy gene in T. brucei (Fig. 3), we could investigate its cellular function by attempting to create CYC2-deficient mutants through gene disruption. These disruption cassettes comprised PAC or BLE antibiotic resistance genes flanked by 5' and 3' regions of the poly(ADP-ribose) polymerase gene and the N-terminal and C-terminal portions of the CYC2 cDNA (see Fig. 8A). For this CYC2 disruption study we used wild type STIB 247 trypanosomes, because this strain has been shown to be diploid by genetic analysis (37). Wild type procyclic STIB 247 trypanosomes were transfected with either the PAC- or BLE-disruption cassette to produce CYC2 heterozygotes (∆cy2::BLE and ∆cy2::PAC). Subsequently, the ∆cy2::BLE cells were transfected with the PAC-disruption cassette, and cells resistant to phleomycin and puromycin were cloned.

The presence of the CYC2 gene and the disruption cassettes in chromosomal DNA of wild type and transfected procyclic cells was investigated by PCR analysis using the primers OL446 and OL243 (Fig. 8B), which correspond to sequences at the ends of the CYC2 cDNA. These sequences are present within the two disruption cassettes as well (Fig. 8A). This PCR on chromosomal DNA of wild type trypanosomes (B, lane 1) resulted in a single DNA fragment of 600 bp, which corresponds to the product generated from the intact CYC2 gene. The same PCR on ∆cy2::PLE cells (B, lane 2) generated two fragments, corresponding to the CYC2 gene (600 bp) and CYC2, in which the BLE gene had integrated (1.6 kb). The same PCR on the ∆cy2::PAC cells resulted in two fragments, corresponding to the CYC2 gene (600 bp) and CYC2, in which the PAC gene had integrated (1.8 kb). PCR analysis on four double-resistant clones that were transfected with both disruption cassettes (B, lanes 4–7) resulted in three fragments corresponding to the CYC2, ∆cy2::PLE, and ∆cy2::PAC. These results suggest that the double-resistant cells had integrated both disruption cassettes in their genome but maintained at least one copy of the CYC2 gene. The presence of the two disruption cassettes and the intact CYC2 gene in the double-resistant clones was confirmed by Southern blot analysis (not shown).

To investigate the site of integration of the disruption cassettes, we performed PCR analysis with primer OL434, which is specific for a sequence located upstream of the CYC2 ORF,
that is not present in the CYC2 disruption cassettes. PCR on DNA of wild type cells (C, lane 1) generated a single fragment (950 bp), which corresponds to the intact CYC2 gene. The same PCR on chromosomal DNA from the heterozygote cells, Δcy2::BLE and Δcy2::PAC (lanes 2 and 3, respectively), each generated two fragments corresponding to the intact CYC2 gene (950 bp) and CYC2, in which the BLE or PAC gene had been integrated (1.8 and 2 kb, respectively). This demonstrates that both disruption cassettes integrated at the correct locus in the cells that were transfected with only one disruption cassette. The same PCR on the double-resistant cells (C, lanes 4–7) resulted in only two bands, corresponding to the normal CYC2 gene (950 bp) and the gene in which the BLE gene had integrated (1.8 kb), whereas no fragment could be detected of 2 kb, corresponding to the gene in which the PAC gene had integrated. This result suggested that the PAC disruption cassette had not integrated at the correct locus when the first allele of CYC2 already had been disrupted, although it did disrupt CYC2 in wild type cells (C, lane 2). This was confirmed by further PCR analysis using the same primer OL434, specific for the sequence upstream of the CYC2 open reading frame (Fig. 8A) in combination with an antisense primer specific for either the BLE gene (OL11) or the PAC gene (OL15). Using the BLE-specific primer, this PCR generated a single 1.4-kb fragment in cells transfected with the BLE disruption cassette and in the cells transfected with both disruption cassettes, showing that the BLE disruption cassette had integrated at the proper CYC2 locus (not shown). PCR carried out with the PAC-specific antisense primer (OL15) generated a single band (Fig. 8D) of 0.85 kb only in Δcy2::PAC cells (D, lane 3), whereas no fragment was detectable in double-resistant cell lines (D, lanes 4–7). In addition, analysis of the DNA content of the double-resistant cells by fluorescence-activated cell sorter showed that they had the same ploidy as wild type trypanosomes (not shown). Hence, these results show that although both disruption cassettes can integrate at the proper CYC2 locus to disrupt CYC2, in heterozygotes in which one CYC2 allele has already been disrupted, the next disruption cassette integrates at a different locus. These results suggest that disruption of both CYC2 alleles results in nonviable cells, which is evidence for an essential function of the CYC2 gene in procyclic T. brucei cells.

**DISCUSSION**

This study has identified two *T. brucei* genes, CYC2 and CYC3, that complement the yeast G1 phase mutant DLI. This mutant is conditional for expression of CLN cyclins, which are essential for cell cycle commitment during G1 (see 6). The DLI mutant, however, is promiscuous in the type of heterologous cyclin that can restore CLN function, and several classes of cyclins from a variety of organisms have been identified using a genetic screen with this mutant. Some of these cyclins function in G1, for example cyclin C (25, 26), which binds CDK8 and interacts with the large subunit of RNA polymerase II (50, 51), and cyclin D1 (52), whereas others were found to function at other phases of the cell cycle, such as the mitotic cyclin CYC1bAt from *Arabidopsis* (27). Therefore, *T. brucei* CYC2 and CYC3 do not necessarily have to fulfill a similar role at START as the CLN cyclins in yeast.

The cyclin box of CYC2 has the most sequence identity to yeast cyclins that function in transcriptional regulation of enzymes involved in phosphate metabolism, the *N. crassa* PREG1 regulatory protein (41), and *S. cerevisiae* PHO80 (54). PHO80 forms a complex with its CDK partner PHO85 and phosphorylates the transcription factor PHO4 in response to changes in phosphate concentration in the cell (42, 55). Recently it has been shown that pyrophosphatase is a major store of inorganic phosphate and is more abundant than ATP in trypanosomes (56). The pyrophosphatase is located in part in an unusual organelle, the acidojalisosome (57, 58). Little is known about the regulation of phosphate metabolism in trypanosomes, but since many cyclins have been identified in yeasts that form CDK complexes which fulfill cellular functions separate from control of cell cycle progression (44), CYC2 could be involved in non-cell cycle-specific functions such as the regulation of phosphate levels. The transcriptional machinery in trypanosomes is unusual as genes are transcribed as polycistronic precursor mRNAs that are subsequently processed by trans-splicing and polyadenylation (59, 60). No polymerase II promoters or transcription factors that might interact with them have been identified in trypanosomes to date, but a major regulatory mechanism for control of gene expression occurs at the post-transcriptional level through mRNA stability (48, 61, 62). Many CDK complexes have been shown to be involved in transcriptional regulation in yeast and higher eukaryotes (24, 64–66). CDKs may also be involved in post-transcriptional control (67, 68). Although some caution is required interpreting subcellular localization with epitope-tagged proteins, the finding...
that CYC2 is not located in the nucleus but in the cytoplasm (Fig. 7) raises the intriguing possibility that trypanosome CDK complexes are involved in control of gene expression at the post-transcriptional level in the cytosol.

In contrast to CYC2, the cyclin box of CYC3 demonstrates the highest level of sequence identity with those of the mitotic S. cerevisiae cyclins CLB1 and CLB2 and with the cyclin box of many other B-type cyclin genes from a variety of species. The sequence identity, though, is low (about 35% over the cyclin box), making it difficult to extrapolate a function for CYC3 based on sequence homology alone. CYC3 does contain a motif with some sequence identity to the destruction box motif, which is typical for mitotic cyclins (12, 69). One difference is that the putative destruction box of CYC3 is located near the C terminus of the protein (Fig. 2), whereas the destruction box in CLB1–4 and in the mammalian A- and B-type cyclins is located at the N terminus (12, 70). However, the position of the destruction box is not important, as fusion proteins with the destruction box at various positions are all degraded in a similar way (12, 46).

The biochemical features of CYC2 were investigated by the construction of a TY epitope-tagged version of CYC2 that was subsequently expressed under inducible control. CYC2ty could complement the yeast mutant DLI to the same extent as CYC2 (Fig. 4), it bound to the Leishmania CDK binding factor p12\text{k}k1, and associated with an active histone H1 kinase (Fig. 5). Furthermore, CYC2 associated with the cdc2-related kinase, CRK3 (Fig. 6), showing the presence of the first cyclin-kinase complex in trypanosomes. Not all cyclins show cell cycle-dependent degradation (71); however, CYC2ty was found to have a short half-life in vivo of less than 4 h, and its degradation was sensitive to the proteosome inhibitors MG132 and lactacystin. \textsuperscript{4} This correlates with the high turnover rate of cyclins in other organisms by proteosome-mediated hydrolysis (72).

The CYC2 disruption studies demonstrated that two distinct knockout cassettes could each disrupt a CYC2 allele in wild type cells but that sequential rounds of disruption with the two cassettes did not produce a null mutant (Fig. 8). These data strongly suggest that CYC2 is essential for the viability of procyclic trypanosomes and that loss of CYC2 cannot be compensated by the function of other T. brucei cyclins. This contrasts the situation in budding yeast where all of the cyclins are involved in control of gene expression at the transcriptional level (53), and we have detected significant monomer activity, as assessed by gel filtration chromatography, in the leishmanial CRK3. \textsuperscript{5} In addition, recombinant CRK1 of the South American trypanosome T. cruzi is highly active in the absence of a cyclin partner (63). If the T. brucei p13\text{ty} binding CRK3 is associating with another cyclin, it is unlikely to be CYC3 since TY-tagged CYC3 does not bind p13\text{ty} (data not shown), and CYC3 does not interact with CRK3 in a two-hybrid interaction assay (Fig. 6). Further investigation of cyclin-dependent and cyclin-independent CRK activity in trypanosomes is ongoing.

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Isolation of *Trypanosoma brucei* CYC2 and CYC3 Cyclin Genes by Rescue of a Yeast G1 Cyclin Mutant: FUNCTIONAL CHARACTERIZATION OF CYC2
Jaap J. Van Hellemond, Philippe Neuville, Ralph T. Schwarz, Keith R. Matthews and Jeremy C. Mottram

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