

# Dissociation of Cytokinesis Initiation from Mitotic Control in a Eukaryote†

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Received 11 August 2005/Accepted 17 October 2005

**Cytokinesis is initiated only after mitotic exit in eukaryotes. However, in the insect (procyclic) form of an ancient protist, *Trypanosoma brucei*, a blockade at the G<sub>2</sub>/M checkpoint results in an enrichment of anucleate cells (zoids), suggesting separated regulations between mitosis and cytokinesis (X. Tu and C. C. Wang, *J. Biol. Chem.* 279:20519–20528, 2004). Polo-like kinases (Plks) are known to play critical roles in controlling both mitosis and cytokinesis. A single *Plk* homologue in *T. brucei*, *TbPLK*, was found to be capable of complementing the *Plk* (*Cdc5*) functions in *Saccharomyces cerevisiae*, thus raising the question of how it may function in the trypanosome with cytokinesis dissociated from mitosis. Depletion of *TbPLK* in the procyclic form of *T. brucei* by RNA interference resulted in growth arrest with accumulation of multiple nuclei, kinetoplasts, basal bodies, and flagella in approximately equal numbers among individual cells. There were, however, few zoids detectable, indicating inhibited cytokinesis with unblocked mitosis and kinetoplast segregation. *TbPLK* is thus apparently involved only in initiating cytokinesis in *T. brucei*. Overexpression of *TbPLK* in the trypanosome did not affect cell growth, but 13% of the resulting population was in the zoid form, suggesting runaway cytokinesis. An immunofluorescence assay indicated that *TbPLK* was localized in a chain of likely flagellum attachment zones in the cytoskeleton. In a dividing cell, a new line of such zones appeared closely paralleling the existing one, which could constitute the cleavage furrow. An exposed region of *TbPLK* at the anterior tip of the cell may provide the trigger of cytokinesis. Taken together, our results revealed a novel mechanism of cytokinesis initiation in the trypanosome that may serve as a useful model for further in-depth investigations.**

Cells multiply by duplicating their genetic contents and dividing into two daughter cells. The eukaryotic cell cycle includes sequential G<sub>1</sub>, S, G<sub>2</sub>, and M phases followed by cytokinesis. Control of this progression requires participation of multiple proteins that ensure precise coordination of different events in time and space. One such protein that plays a crucial role in both mitosis and cytokinesis is polo-like kinase (Plk). It is a highly conserved serine/threonine kinase characterized by two polo boxes at the C terminus (18, 35) and has been found in all eukaryotes, from *Saccharomyces cerevisiae* to human (8). Plks play multiple pivotal roles in regulating the G<sub>2</sub>/M transition (1), the metaphase/anaphase transition (2), anaphase release (19), mitotic exit (17), and initiation of cytokinesis (14), and these roles are largely conserved throughout evolution (12). Phosphorylation of the conserved Thr210 in human Plk up-regulates its activity during mitosis (32), whereas polyubiquitination of Plk by the anaphase-promoting complex *cdh1* is required for proteasomal degradation and plays an essential role during the exit from mitosis (30). The single Plks in budding yeast (*Cdc5*) and fission yeast (*Plo1*) are located on the spindle pole bodies from the G<sub>1</sub> phase to late mitosis (27, 38) and are then relocated to the cytokinetic ring structures (3, 33). This dynamic subcellular localization of Plks presages their functional complexity. It also reflects the importance of a close coordination between mitotic exit and initiation of cytokinesis in cell cycle progression.

During the G<sub>2</sub>-to-M-phase transition in *Drosophila melanogaster*, low Plk causes failure in the recruitment of proteins to the spindle pole, resulting in aberrant spindle pole formation (40). In budding yeast, a *cdc5* (*plk*) mutant exhibited cell cycle arrest at multiple stages during mitosis with pairs of stretched spindles that failed to initiate cytokinesis (20). In fission yeast, a *plo1* (*plk*) mutant caused formation of monopolar mitotic spindles and incomplete septation (29), whereas overexpression of *Plo1* led to ectotypic septum formation (41). Thus, depletion of Plk generally leads to mitotic arrest.

The trypanosomes are ancient, unicellular eukaryotes whose life cycles involve a cyclic transmission between the mammalian host and an insect vector, the tsetse fly. Life cycle progression in the two distinct environments is intimately linked to cell cycle regulation of the two replicating forms of *Trypanosoma brucei*, namely, the bloodstream form and the insect (procyclic) form (25). There are in each cell a single flagellum, basal body, nucleus, mitochondrion, and the mitochondrial DNA complex known as the kinetoplast. Each of these organelles is duplicated and segregated into progeny cells during cell division. Like the other eukaryotes, trypanosomes undergo the same periodic nuclear events: the G<sub>1</sub>, S, G<sub>2</sub>, and M phases (47). But there is also a well-coordinated kinetoplast cycle with a discrete S phase starting shortly before the nuclear S phase and a kinetoplast segregation prior to the onset of mitosis (47). A basal body, located next to the kinetoplast across the outer mitochondrial membrane, has the flagellum extended from it toward the anterior end of the cell while being connected to the cell body through an undulating membrane structure (36). A probasal body is formed next to the existing basal bodies. It matures and nucleates the growth of a new flagellum. The new flagellum grows alongside the existing one, and its tip is con-

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† Supplemental material for this article may be found at <http://ec.asm.org/>.

nected to a cytoskeleton-associated flagellum attachment zone (FAZ) in the cell cytoskeleton positioned at the base of the undulating membrane (22). The completion of the daughter flagellum eventually leads to longitudinal cell division following mitotic exit and completion of cytokinesis (22). The location of the cleavage furrow in *T. brucei* has not yet been identified but is presumed to be associated with the plasma membrane alongside the newly formed flagellum.

Using rhizoxin and aphidicolin, Ploubidou et al. (31) indicated that procyclic-form *T. brucei* cytokinesis is not dependent upon either mitosis or nuclear DNA synthesis. Our recent investigations indicated also that when procyclic-form *T. brucei* was arrested in G<sub>1</sub> phase by knocking down the expression of cyclin E1 (23) or *cdc2*-related kinase 1 (CRK1) through RNA interference (RNAi) (42), slender anucleate cells (zooids) were generated in substantial numbers. For the G<sub>2</sub>/M cells arrested by a cyclin B2 (23) or CRK3 knockdown (42), an even higher number of stumpy zooids was produced. This phenomenon suggested a dissociation between the nuclear and kinetoplast cycles; when the former is blocked, the latter can apparently still proceed to drive cytokinesis and cell division to completion. Furthermore, treatment of the procyclic form with the phosphatase inhibitor okadaic acid (9) inhibited kinetoplast segregation and cell division but not mitosis, resulting in multinucleated cells. Taken together, these results suggest a dissociation between the controls of mitosis and cytokinesis that has not yet been observed among other eukaryotes.

The identification of a single-copy *Plk* homologue in *T. brucei* (TbPLK) a few years ago (13) raised the interesting question of how a Plk, known to perform multiple critical roles in controlling both mitosis and cytokinesis, could function properly in procyclic-form *T. brucei*. TbPLK is located on chromosome 7 in *T. brucei* (Gene DB accession no. Tb07.2F2.640) (13). The protein has 47.4% sequence identity with human Plk1 in the N-terminal serine/threonine catalytic domain (nucleotides 43 to 297), with an essential Ser/Thr protein kinase active-site signature (nucleotides 160 to 172) and a protein kinase ATP-binding region signature (nucleotides 49 to 70). The two polo boxes (nucleotides 564 to 642 and 690 to 756) at the C terminus have 30% sequence identity with those of the human Plk (see Table S1 in the supplemental material). A large insertion is present at the C terminus of TbPLK, which is absent from mammalian Plks (13). There is also in TbPLK a polyasparagine sequence (nucleotides 414 to 431), which is absent from the Plks of other organisms, showing length polymorphisms among various *T. brucei* isolates (13).

In the present study, we demonstrate that TbPLK is not involved in the regulation of either mitosis or kinetoplast segregation but plays a role only in initiating cytokinesis in procyclic-form *T. brucei*. Overexpression of TbPLK led to zoid formation, whereas immunofluorescence assays localized TbPLK in a chain of likely FAZs, implying an involvement of TbPLK with cell division.

#### MATERIALS AND METHODS

**Complementation of the *Saccharomyces cerevisiae* temperature-sensitive *cdc5-1* mutant.** The full-length gene sequence of TbPLK was amplified by PCR and subcloned into the vector pGADT7 (Invitrogen). This vector was modified by adding an additional NdeI site at position 1480 to remove the fragment from positions 1480 to 1964 by NdeI digestion. The temperature-sensitive yeast *cdc5-1*

mutant (6) and the vector containing the full-length gene sequence for yeast Plk (Cdc5) under endogenous-promoter control were kindly provided by Kevan Shokat of UCSF. The yeast *cdc5-1* mutant was transfected with the construct containing TbPLK, and the transfectant was selected on Leu-deficient plates and then restreaked on yeast extract-peptone-dextrose (YPD) agar plates. Cells were then allowed to grow on the plates at 25°C or 37°C for 3 days.

**Cell culture.** Procyclic-form strain 29-13 cells (46) were grown at 26°C in Cunningham's medium (7) supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA). Antibiotics G418 (15 µg/ml) and hygromycin B (50 µg/ml) were included in the culture medium to maintain the T7 RNA polymerase and tetracycline repressor gene constructs in the cells.

**RNAi.** A fragment (nucleotides 85 to 432) of the TbPLK cDNA (GenBank accession number Y13968) was amplified by PCR using a pair of gene-specific primers that introduced restriction enzyme sites XhoI and HindIII at the ends of the PCR product (sequences available upon request). After digestion with the two restriction enzymes, the PCR product was ligated to the XhoI/HindIII-digested pZJM vector (45). The resulting RNAi construct was linearized with NotI for integration into the *T. brucei* ribosomal intergenic region upon introduction into *T. brucei* strain 29-13 by electroporation as described previously (24). The transfectants were selected on agarose plates containing 2.5 µg/ml phleomycin, and individual cells were cloned by limiting dilutions (5). Tetracycline (1 µg/ml) was added to induce transcription of the DNA insert from the T7 promoter. The double-stranded RNA (dsRNA) thus synthesized is expected to lead to specific degradation of its corresponding mRNA in *T. brucei* (4, 26). To evaluate the effects of each RNAi on cell proliferation, cells in a growing culture were counted at different times with a hemocytometer and data were plotted on a logarithmic scale.

**Semiquantitative RT-PCR.** Total RNA was extracted from *T. brucei* procyclic-form cells with TRIZOL reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Roche, Indianapolis, IN) to remove DNA. Semiquantitative reverse transcriptase (RT) PCR was then performed on the RNA template using the one-step RT-PCR kit (Invitrogen, Carlsbad, CA) and a pair of gene-specific primers that differ from the primer pair used in generating the original RNAi construct (sequences available upon request).

**FACS analysis.** Propidium iodide (PI)-stained cell samples for fluorescence-activated cell sorting (FACS) were prepared and analyzed according to the procedure described previously (42). The stained cell samples from FACS analysis were also examined with an Olympus phase-contrast and fluorescence microscope (Melville, NY) to tabulate the numbers of nuclei and kinetoplasts in individual cells from a population of 200.

**Live-cell imaging.** To visualize the living cells, cells in a 96-well tissue culture plate were treated with the living fluorescent dye SYTO Green Fluorescent (S7572; Molecular Probes), which specifically stains nucleic acids. Time-lapse videos were made with an inverted fluorescence microscope (magnification, ×40), and Metavue software was used for analysis.

**Immunofluorescence microscopy.** For the immunofluorescence experiments, cells were harvested, washed three times in phosphate-buffered saline (PBS), and fixed with 3.7% formaldehyde for 5 min. They were then washed in PBS, loaded onto poly-L-lysine-coated cover slides (Sigma) for 20 min, washed again, and blocked in a blocking buffer (2% bovine serum albumin and 0.1% Triton X-100 in PBS) at room temperature for 60 min. Cells were washed and incubated with the primary antibody for 1 hour, washed, and then further incubated with the secondary antibody. The various antibodies used in this study were YL1/2, for staining the basal body (a rat monoclonal antibody [MAb] against yeast tyrosinated α-tubulin, used at a 1:400 dilution; Chemicon, Temecula, CA); L8C4, for flagellum staining (a mouse MAb against paraflagellar rod [PFR 2] protein, used at a 1:4 dilution [10]; Keith Gull, Oxford University); KMX-1, for mitotic spindles (a mouse MAb against *Physarum polycephalum* amoebal tubulin protein [34]; Keith Gull, Oxford University); and a hemagglutinin (HA) probe, for the triple HA tag (sc-7392; Santa Cruz Biotech). The various secondary antibodies used were anti-rat Alexa Fluor 488 (A-21208; Molecular Probes), anti-mouse Alexa Fluor 488 (A-21422; Molecular Probes), and anti-mouse Alexa Fluor 588 (A-21422; Molecular Probes). Following incubation with the secondary antibodies, cells were washed and the nuclei and kinetoplasts were stained with 1 µg/ml of 4',6'-diamidino-2-phenylindole (DAPI). The slides were mounted on Vectashield and examined with a fluorescence microscope (Olympus IX70).

All photographs were taken with an Olympus PlanApo 60× (numerical aperture, 1.4) Oil Ph3 objective lens or with a 100× (numerical aperture, 1.3) oil lens. Data were collected with a Photometrics Cool Snap HQ charge-coupled-device camera. Photographs were processed with MetaVue software, version 5.0, and then with Adobe Photoshop CS.

**Overexpression of TbPLK-3HA in procyclic-form *T. brucei*.** The full-length gene TbPLK was amplified by PCR using primers that had a HindIII site at the

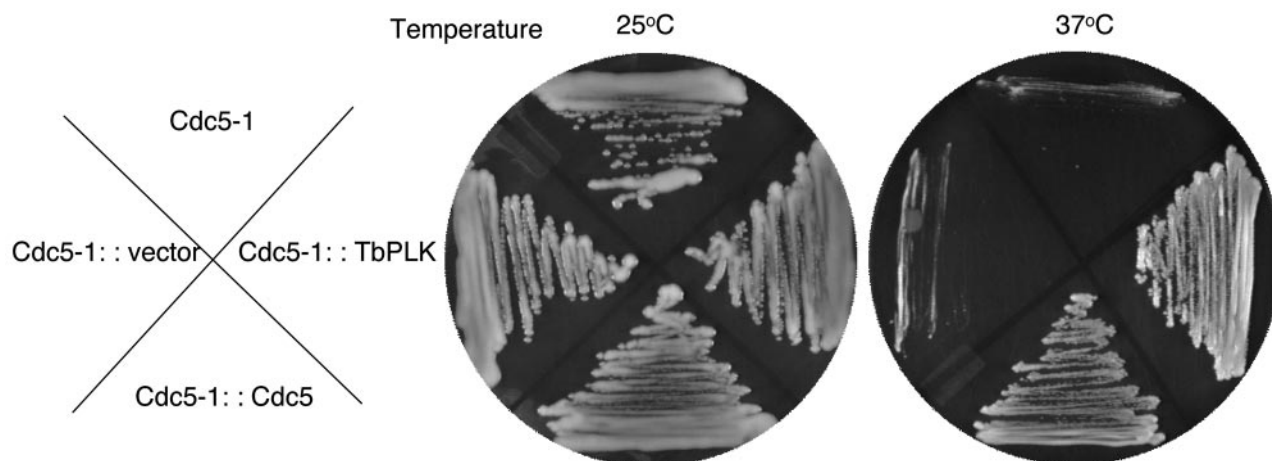


FIG. 1. Complementation of the *S. cerevisiae* Cdc5-1 defect by ectopic expression of TbPLK. A temperature-sensitive *S. cerevisiae* mutant (*cdc5-1*) was transfected with the full-length gene of TbPLK and yeast *Cdc5*, with the empty vector as negative control. The transfected cells were streaked on YPD agar plates and incubated for 3 days at either 25°C or 37°C.

5' end and an XhoI site at the 3' end of the gene. The reverse primer also contained the sequence of a triple HA tag (3HA) at the C terminus of TbPLK in frame with the gene sequence. PCR-amplified TbPLK-3HA was cloned into the HindIII/XhoI-digested pLew100 vector (46), which places expression of the inserted gene under the control of a tetracycline-inducible T7 promoter. The cloned construct was linearized with NotI and transfected into procyclic-form *T. brucei* cells (46). Stable transfectants were selected under phleomycin. Expression of TbPLK-3HA was induced by adding 0.1 µg/ml of tetracycline to the culture medium.

**Preparation of cytoskeleton.** Cells were harvested by centrifugation and washed three times in PEME buffer {100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 2 mM EGTA, 0.1 mM EDTA, and 1 mM MgSO<sub>4</sub>}. Pellets after the third wash were suspended in PEME containing 1% NP-40 detergent, incubated for 5 min at room temperature, and rewashed in PEME for cytoskeleton preparation. For high-salt treatment, the cytoskeleton preparation was further incubated in PEME containing 1% NP-40 and 1 M NaCl. Both preparations were stored at -20°C until needed.

## RESULTS

**TbPLK complements the Cdc5 function lost in the *Saccharomyces cerevisiae* *cdc5-1* mutant.** In order to check if TbPLK is a functional homologue of yeast Cdc5, we introduced the TbPLK gene into the yeast *cdc5-1* temperature-sensitive mutant. The cells were streaked on YPD agar plates and incubated at 37°C for 3 days. The results indicated that both the TbPLK gene and the yeast *Cdc5* gene complemented the missing functions of Cdc5 in the yeast *cdc5-1* mutant to the same extent as the endogenous *Cdc5* gene (Fig. 1) but that the empty vector and the *cdc5-1*-transfected cells failed to grow at the restrictive temperature (Fig. 1). TbPLK is thus both a structural and a functional homologue of Cdc5.

**Depletion of TbPLK from the procyclic form of *T. brucei*.** To determine the potential roles of TbPLK in the procyclic form of *T. brucei*, mRNA encoding the protein was depleted from these cells by using the RNAi technique. A cDNA fragment from the unique coding region of the TbPLK gene (nucleotides 85 to 432) was synthesized by RT-PCR and inserted into the tetracycline-inducible RNAi vector pZJM between two opposing T7 promoters and two tetracycline operators (45). The linearized construct was introduced into the procyclic-form *T. brucei* strain 29-13 by electroporation, and the cloned trans-

fectant cell line was induced by tetracycline to synthesize the dsRNA encoded by the cDNA insert. The dsRNA is expected to degrade the corresponding mRNA through the machinery of RNAi in *T. brucei* (45).

To verify the effect of RNAi on the level of TbPLK mRNA, semiquantitative RT-PCR was performed on the RNA samples extracted from tetracycline-induced cells. While the level of TbPLK mRNA remained relatively unchanged in the uninduced cells, it was decreased significantly in cells 3 days after RNAi induction (Fig. 2, inset). The  $\alpha$ -tubulin mRNA, included as a sampling control, demonstrated no detectable difference

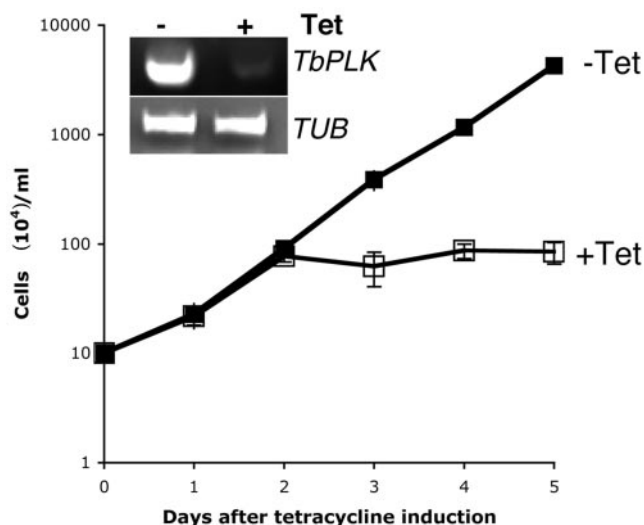


FIG. 2. Effect of TbPLK depletion on the growth of procyclic-form *T. brucei*. Cloned procyclic *T. brucei* cells harboring the TbPLK RNAi plasmid construct were incubated in a culture medium consisting of 1 µg/ml tetracycline (+Tet) or without tetracycline (-Tet). Growth of the cells was monitored daily, and the numbers of cells were plotted on a logarithmic scale. The inset shows the level of TbPLK mRNA on the third day of RNAi measured by semiquantitative RT-PCR on the RNA sample isolated from the cells.  $\alpha$ -Tubulin (TUB) mRNA was included as a sampling control.

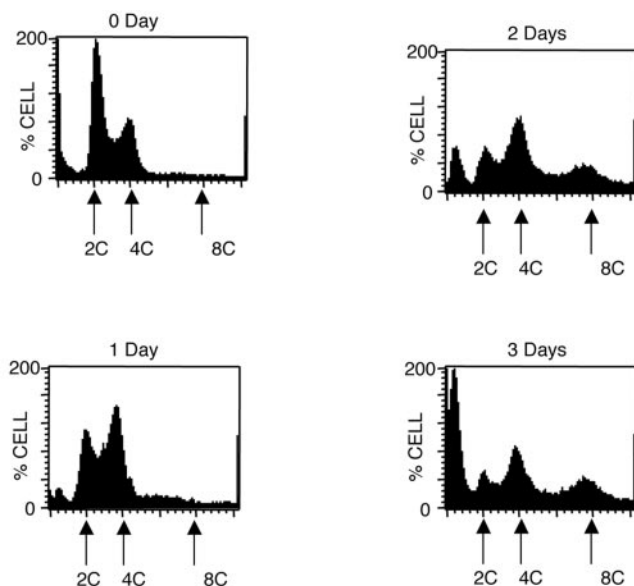


FIG. 3. FACS analysis of TbPLK-depleted cells. *T. brucei* procyclic-form cells under RNAi were sampled each day and stained with PI. Total DNA content in the cell sample was analyzed by FACScan.

between the uninduced and induced cells (Fig. 2, inset). These results indicate that expression of TbPLK was significantly down-regulated by RNAi in the procyclic form of *T. brucei*.

**TbPLK is essential for the growth of procyclic-form *T. brucei*.** The growth of cells with induced RNAi did not differ initially from the uninduced control on the first day but decreased by 15% after 2 days (Fig. 2). It then ceased completely thereafter, with a cell density remaining at approximately eightfold that at the beginning of RNAi, which could represent the result from three cell divisions. Morphology of the cells started to change on the second day of tetracycline induction. Time-lapse video microscopic examination of SYTO Green Fluorescent-stained live cells on the third day of RNAi showed mobile, enlarged cells with multiple nuclei and multiple flagella (see Video S1 in the supplemental material). These cells were moving randomly in the culture medium because their multiple flagella were apparently not beating synchronously. In some cells, flagella were apparently not properly attached to the cytoskeleton.

**TbPLK-depleted cells continue with DNA synthesis without cell division.** To further characterize the growth defect upon TbPLK depletion from the procyclic form of *T. brucei* and to understand the potential role of TbPLK in cell cycle regulation, FACScan analysis was performed on PI-stained RNAi cells. The data, presented in Fig. 3, indicate a steady decrease in the population of 2C cells throughout the 3 days of RNAi. There was an appreciable increase in 4C cells after 1 day of RNAi, but these cells were gradually replaced by 8C cells on days 2 and 3, suggesting continued synthesis of DNA in individual cells without cell division. These results agree with the previous observation from our time-lapse video that the RNAi cells were mostly multinucleated on day 3. These cells were apparently unable to survive much beyond 2 to 3 days, as the cell population in the sub-G<sub>1</sub> phase began to rise significantly on days 2 and 3 (Fig. 3). As revealed by microscopic examina-

tion, these were apoptotic cells. No zoids were identified in this population (data not shown).

**Kinetoplast segregation and nuclear division continue in TbPLK-depleted *T. brucei*.** To ensure that the TbPLK-depleted cells are indeed multinucleated, we stained the cells with DAPI and visualized them with a fluorescence microscope. The results presented in Fig. 4A show that the RNAi cells are mostly multinucleate as well as multikinoplast after 3 days of induction. A more thorough and careful tabulation of various cellular morphologies throughout the 3-day RNAi indicated a dramatic decrease in the numbers of cells with a single nucleus and a single kinetoplast (1N1K), from 72.7 to 3.3% of the population (Fig. 4B). This change was accompanied by a drastic increase in the numbers of cells with multiple (more than two) nuclei and multiple (more than two) kinetoplasts (XNXX), from 0.7 to 93.3%. In between, the numbers of 1N2K cells were reduced from 13.3 to <0.5%, while the numbers of zoids (0N1K) were also reduced, from 3.3 to <0.5%. The numbers of 2N2K cells increased from 7.3 to 29.0% during the first day of RNAi but were reduced to 1.7% after 3 days, whereas the number of 2N1K cells increased from 0.8 to 14.0% during the 1st day but was reduced to 0.5% on day 3. Taken together, the data suggest that the TbPLK-depleted procyclic-form *T. brucei* cells continue with nuclear division and kinetoplast segregation without cell division. Apparently, only cytokinesis cannot proceed in the TbPLK knockdown cells.

**Depletion of TbPLK impairs cytokinesis.** The kinetoplast in *T. brucei* is physically attached to the mature basal body through a tripartite attachment complex across the mitochondrial membrane (28), and the basal body serves primarily as an anchor and a nucleus of growth for the flagellum. We had observed in the time-lapse video that the TbPLK-depleted cells appeared to have multiple nuclei and multiple flagella. To verify this observation and to investigate if the multiple flagella are associated with corresponding basal bodies, which are in turn connected to the multiple kinetoplasts, we performed immunostaining for flagella and basal bodies in DAPI-stained TbPLK-depleted cells. The results, presented in Fig. 5, show approximately four nuclei, four kinetoplasts, four basal bodies, and four full-length flagella in a single TbPLK-depleted cell. Thus, in addition to continued nuclear division and kinetoplast segregation, the corresponding basal body multiplication and growth of individual flagella to their full lengths also occur in TbPLK-depleted cells. The multiplications of these organelles are apparently well coordinated, like those in wild-type *T. brucei*. Cell cycle progression is thus most likely arrested in the early phase of cytokinesis after the completion of kinetoplast and basal body duplication, mitosis, and new flagellum synthesis.

**Mitotic spindle assembly proceeds normally in TbPLK-depleted *T. brucei*.** Plk has been known to play a major role in mitotic spindle assembly in eukaryotes (12). Depletion of Plks from those organisms invariably leads to spindle defects (12). To verify if TbPLK depletion exerts any effect on mitotic spindle assembly in procyclic-form *T. brucei*, we labeled the mitotic spindle in the TbPLK-depleted cells with KMx-1 (a mouse MAb against *P. polycephalum* amoebal  $\beta$ -tubulin protein; used at a 1:50 dilution), which preferentially recognizes  $\beta$ -tubulin in the mitotic spindles (34). Analysis of the KMx-1- and DAPI-stained cells with a fluorescence microscope revealed that

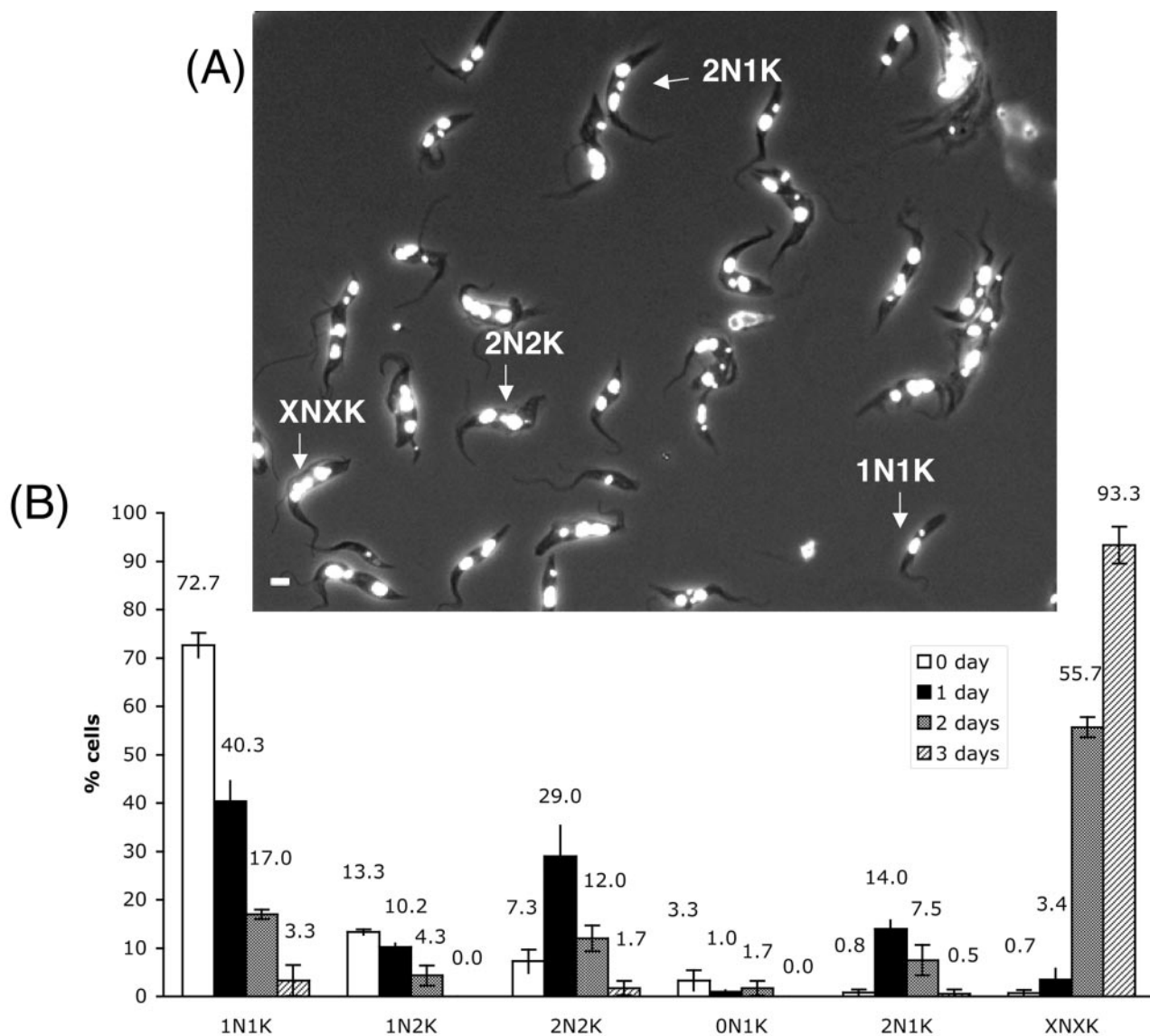


FIG. 4. Phenotypic analysis of TbPLK-depleted cells. (A) Cells after 2 days of RNAi were stained with DAPI and observed with an inverted fluorescence microscope. Bar, 5  $\mu$ m. (B) DAPI-stained time samples of cells under RNAi were scored for numbers of nuclei and kinetoplasts in each cell. Data are presented as mean percentages  $\pm$  standard errors of total cells counted (>200) from three independent experiments. The numbers above the bars represent percentages of the cell population.

many of the cells with four nuclei had double mitotic spindles (Fig. 6). Some of the pairs of spindles are relatively short, suggesting that the cells are in metaphase (Fig. 6A), whereas the others demonstrate stretched double mitotic spindles, indicating that the cells are probably in late anaphase (Fig. 6B). These spindle structures have normal appearances, as in the control cells (34). Furthermore, there is also a significant percentage of the XNXK cell population showing no evidence of spindle-like structures (Fig. 6A). Those are cells that had probably already exited mitosis, suggesting that the TbPLK-depleted cells are not trapped in the mitotic phase but rather are spread throughout the cell cycle. This provides yet more supporting evidence that TbPLK depletion does not affect mitosis in procyclic-form *T. brucei*.

**Overexpression of TbPLK in procyclic-form *T. brucei* increases the zoid population.** The full-length TbPLK gene with a triple HA tag at the C terminus was cloned into the *T. brucei* expression vector pLew100 (46), which placed the gene under the control of a tetracycline-inducible promoter. Procyclic-form *T. brucei* cells transfected with the construct were selected under phleomycin and subjected to single-cell cloning by limiting dilution. Expression of TbPLK-3HA in the cloned cells was then induced with tetracycline (0.1  $\mu$ g/ml). After 5 days of induction, semiquantitative RT-PCR showed a significantly enhanced level of TbPLK mRNA (Fig. 7A, upper inset), and Western blotting indicated the expression of TbPLK-3HA protein in the transfected cells (Fig. 7A, lower inset).

Growth of the tetracycline-induced cells was then moni-

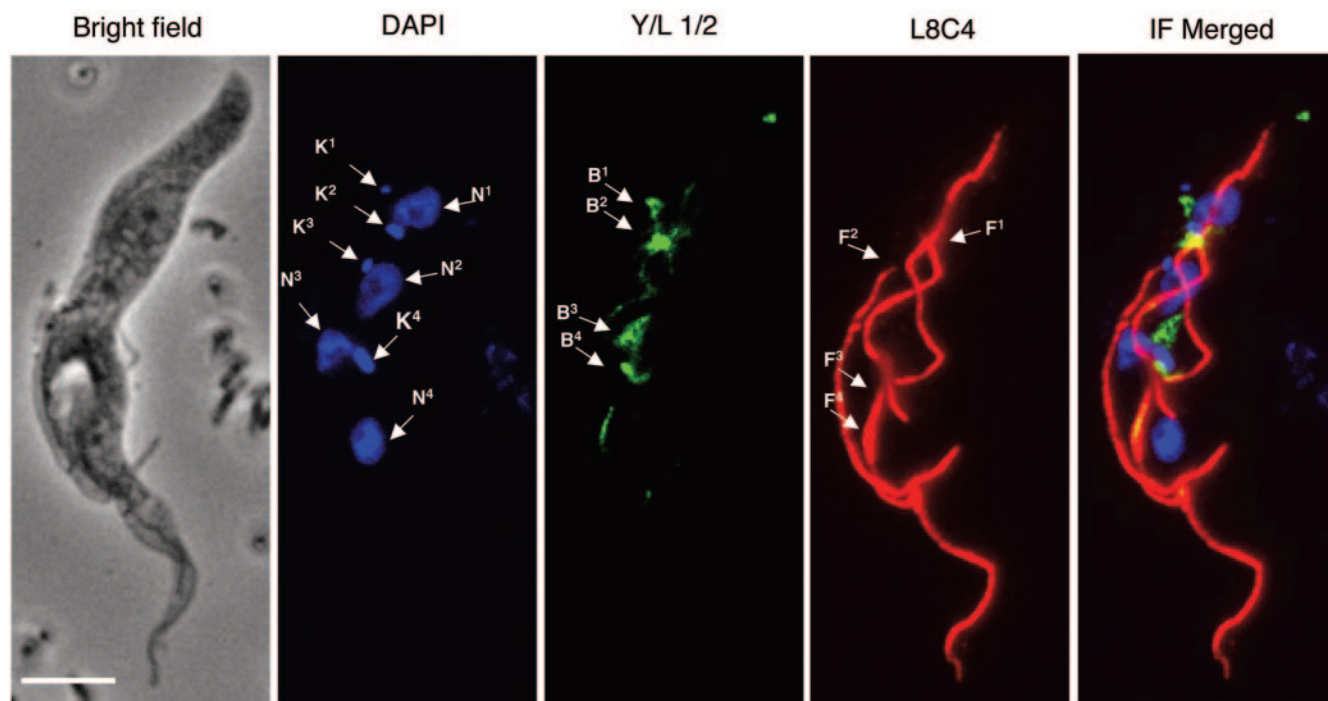


FIG. 5. Triple labeling of a TbPLK-depleted cell. A procyclic-form *T. brucei* cell 2 days into RNAi was stained with DAPI for nuclei (N) and kinetoplasts (K) (blue), with YL1/2 for basal bodies (B) (green), and with L8C4 for flagella (F) (red). IF, immunofluorescence images. Bar, 5  $\mu$ m.

tored, and the result turned out to be quite similar to that with uninduced cells (Fig. 7A). There was neither a stimulatory nor an inhibitory effect on cell growth from overexpression of TbPLK. When the cells were classified by the numbers of nuclei and kinetoplasts after 5 days of induction, however, there was a decrease in the 1N1K population from 80 to 66% accompanied by a 0.6 to 12.6% increase in zoids (Fig. 7B). Apparently, TbPLK overexpression accelerated the paces of cytokinesis and cell division following kinetoplast segregation, which is usually completed prior to mitotic exit (31). Thus, among a substantial number of cells, cytokinesis may have taken place before the completion of mitosis, resulting in zoid formation. This observation provides yet another indication that TbPLK is not involved in controlling mitotic exit. Mitosis and cytokinesis can be easily dissociated in trypanosomes.

**Intracellular localization of TbPLK.** To study the intracellular localization of TbPLK, a double immunofluorescence assay of DAPI-stained TbPLK-3HA-expressing cells was carried out with the YL1/2 antibody against the basal body and the HA probe against TbPLK-3HA. The latter appeared in a well-defined small region at the tip of the anterior end of 1N1K cells (Fig. 8, top panels). This is the distal anterior tip of the cell where cell division is initiated. It is also where we consider the FAZ to be most likely located in cells with mature flagella (see below). The FAZ provides the structural information for the growing daughter flagellum. During its synthesis, the tip of the flagellum travels from the posterior to the anterior end of the cell along the dorsal side of the cell body under the undulating membrane structure that connects to the mother flagellum (21). However, the detailed mechanism of FAZ action remains unclear. In 1N2K and 2N2K cells which were about to divide (Fig. 8, middle and bottom panels), however, TbPLK

appeared in two well-defined and separated small areas, with one of the two located at the anterior end of the cell, as seen in the 1N1K cell. The other TbPLK-concentrated region was located in the midportion of the cell, without an obvious connection with the basal bodies and kinetoplasts (Fig. 8, middle and bottom panels). However, the bright-field photograph indicates that it is located at the tip of the second flagellum, presumably in the newly formed FAZ associated with the second flagellum synthesis. Though the data did not reveal whether the signal for flagellum growth is at the end of the cell or the end of the flagellum, the observation provided a strong indication that TbPLK may be localized in the FAZ. It also raised the question of whether this TbPLK-containing FAZ represents a zone moving along the dorsal side of the cell cytoskeleton and guiding flagellum synthesis or whether there is a chain of individual FAZs formed during the synthesis of the flagellum which remains underneath the membrane structure of the cell for guiding the parallel formation of a new chain of FAZs for the synthesis of a daughter flagellum prior to cell division. The second possibility seems more appealing if one assumes that the primary function of the FAZ is preserving the cell morphology of trypanosomes.

**TbPLK is apparently localized in a chain of FAZs.** The FAZ is known to contain four specialized microtubules associated with the endoplasmic reticulum and the electron-dense filaments (36). It is tightly bound to the cytoskeletons of trypanosomes. If TbPLK is localized in the FAZ, it could also be closely associated with the cytoskeleton. Thus, in an attempt to verify if this is so, we prepared the cytoskeleton with detergent treatment and subjected it to an immunofluorescence assay. A single concentrated region of TbPLK was found still localized at the anterior tip of the cytoskeleton of 1N1K cells. However,

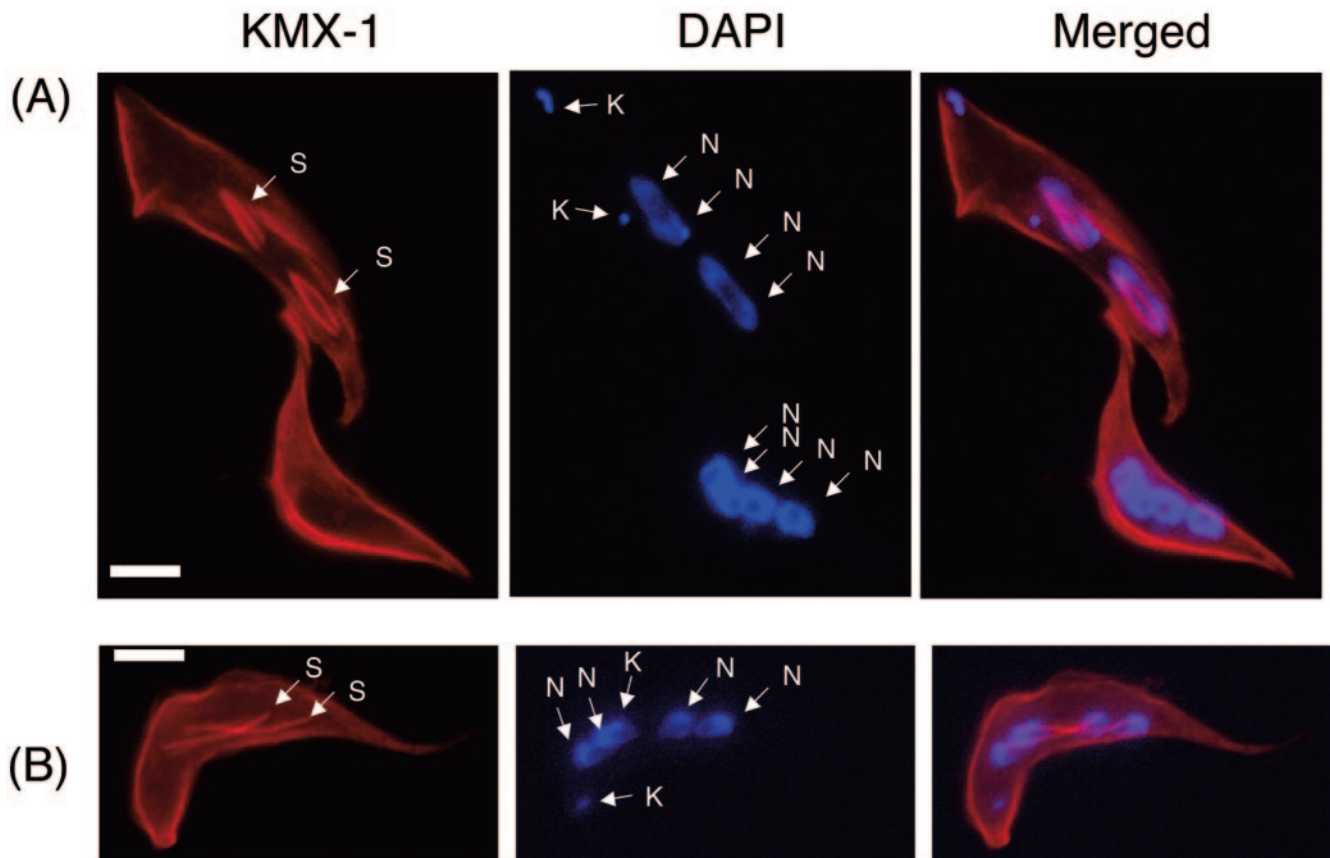


FIG. 6. Mitotic spindle labeling in TbPLK-depleted cells. Cells 2 days into RNAi were fixed with 3.7% formaldehyde and labeled with KMX-1 and DAPI. (A) Two tetranucleated cells. The one above has two short mitotic spindle structures (S) each mediating a pair of nuclei (N), suggesting the state of metaphase. The one below shows no spindle structure. (B) Tetranucleated cell with two elongated spindle structures each connected to a pair of nuclei. It suggests that the cell may be in late anaphase. K, kinetoplast. Bars, 5  $\mu$ m.

the protein was also distributed in a single punctate chain extending all the way from the posterior basal body and kinetoplast to the anterior end of the cytoskeleton along the dorsal side of the cell (Fig. 9A, left). This line of distribution, which follows the path of the flagellum and is located underneath it as if representing a track of multiple FAZs, is apparently buried beneath the plasma membrane and escaped detection in the previous experiment until the membrane structure was removed by detergent. This is apparently a novel finding. Due to the lack of a suitable marker for the FAZ in the previous study (36), identification of the FAZ was limited when electron microscopy was used. It was able to show only the cross section of an FAZ associating the flagellum with the cytoskeleton on the dorsal side of the cell. With TbPLK as a potential marker for FAZ, we now realize that the latter exists likely in a chain with only the one at the very anterior tip exposed.

Two closely parallel punctate lines of TbPLK were observed in the cytoskeleton of a 1N2K cell (Fig. 9A, right). The longer line is very similar to the one observed in the 1N1K cytoskeleton. The shorter one originated from the second basal body and kinetoplast and extended along the longer line for a relatively short distance to the midportion of the cell. This could be the second line of FAZs guiding the formation of a daughter flagellum. This second line of FAZs may begin to form only after the duplication of the kinetoplast and basal body, because

it was not found in 1N1K cells and was only halfway completed in 1N2K cells. By being closely associated with the first FAZ line in the cytoskeleton, this second FAZ line may both direct the synthesis of a daughter flagellum and transfer the original cellular morphology to the daughter cell.

The FAZ is apparently so tightly associated with the cytoskeleton that a high-salt treatment in the presence of detergents is known to remove all other cytoskeleton components except the four specialized microtubules, the flagellum, and probably also the FAZ (36). To investigate if TbPLK is also closely associated with the four specialized microtubules that could sustain such harsh treatment, we treated the cytoskeleton with high levels of salt and subjected it to an immunofluorescence assay. The outcome indicated that the punctate line of TbPLK still remained intact and associated with a fiberlike structure, which could be the four specialized microtubules (Fig. 9B). There is thus apparently a tight association between TbPLK and the four specialized microtubules identified in the FAZ. This provides a strong indication that TbPLK is localized in the FAZ, which forms a chain of distribution on the dorsal side of the cytoskeleton.

## DISCUSSION

Previous cell cycle analyses of the procyclic form of *T. brucei* have revealed that cytokinesis and cell division can proceed to

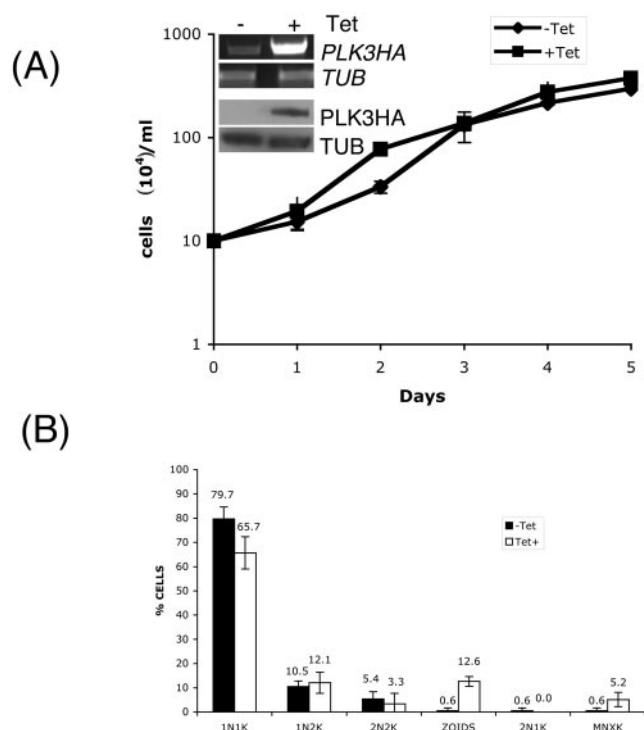


FIG. 7. Overexpression of TbPLK-3HA. (A) TbPLK-3HA expression was induced by adding 0.1  $\mu$ g/ml tetracycline (Tet) to the culture medium. Cell samples were collected 5 days thereafter, and total RNA and protein prepared from the cells were analyzed and quantitated by semiquantitative RT-PCR (top inset) and Western blotting (bottom inset), respectively. Samples of cells were counted and plotted versus time. (B) Cells harvested after 5 days of induction were fixed and stained with DAPI for numbers of nuclei and kinetoplasts in individual cells. Data are presented as mean percentages  $\pm$  standard errors of total cells counted (>200) from three independent experiments. The numbers above the bars represent percentages of the cell population.

completion in cells arrested in G<sub>1</sub> phase and G<sub>2</sub>/M phase, resulting in the production of large numbers of anucleate zoids (23, 42, 43). Cytokinesis and division in these cells are thus likely driven by the kinetoplast-basal body segregation cycle. However, the detailed mechanism by which this cycle regulates cytokinesis is not known. In the current study, we demonstrated that the only Plk homologue in *T. brucei*, TbPLK, which can complement the functions of Cdc5 in *S. cerevisiae*, is apparently not involved in regulating mitosis or kinetoplast segregation in procyclic-form *T. brucei*. Its depletion does not interfere with nuclear division, kinetoplast segregation, basal body duplication, or even flagellum propagation. The depletion of TbPLK brings the cell cycle to a full stop at a point beyond those events. This point is now tentatively assigned to the initiating phase of cytokinesis, which can be readily dissociated from both mitosis and kinetoplast segregation. The enhanced zoid formation from cells overexpressing TbPLK further illustrates that this kinase is primarily involved in cytokinesis initiation independently of the nuclear and kinetoplast cycles in procyclic-form *T. brucei*.

Initiation of cytokinesis in *S. cerevisiae* is triggered only by mitotic exit, which requires inactivation of Cdc28-C1b (11) by two cooperative pathways: the Cdc14 early-anaphase-release

network (39) and the mitotic-exit network (MEN) (19). Both networks are initiated by the action of Cdc5, and both lead to a release of activated phosphatase Cdc14, which inactivates Cdc28-C1b for mitotic exit. A homologue of Cdc14 was identified in the Trypanosome Genome Database, but a knock-down of its expression in procyclic-form *T. brucei* did not register any detectable phenotype (X. Tu and C. C. Wang, unpublished data). The Cdc14 homologue is thus apparently not required for mitotic exit in *T. brucei*, which suggests that a TbPLK-initiated MEN-like pathway for mitotic exit may not exist in *T. brucei*.

In MEN, Cdc5 phosphorylates and reduces the ability of Bfa1 and Bub2 to interact with the Ras-like GTPase Tem1 (16) and releases the phosphatase Cdc14, which then dephosphorylates Lte1 (39). The latter then activates Tem1, which in turn recruits Cdc15 and initiates a Cdc15-Dbf2/Mob1 kinase cascade that triggers the bulk release of Cdc14 from the nucleolus and brings a complete collapse of cyclin-dependent kinase activities (37). Recently, a Dbf2/Mob1 homologue in *T. brucei* was depleted from the procyclic form (15), resulting in a significant increase in postmitotic cells with a mispositioned cleavage furrow. Apparently, mitotic exit was not affected and cytokinesis was initiated in these cells but disrupted during its progression. Thus, like TbPLK, *T. brucei* Mob1 is most likely involved in only cytokinesis downstream from TbPLK, suggesting a pathway in *T. brucei* regulating only cytokinesis. This is very much like the septation initiation network in fission yeast, where a similar pathway leading from Plo1 to Sid2/Mob1 is involved only in initiating cytokinesis (14). *T. brucei* Mob1 had a punctate distribution throughout the cytoplasm and was excluded from the nucleus during the entire life cycle (15), which reinforces the view that Mob1 is not required for mitotic exit in *T. brucei*.

It has been proposed that the FAZ in *T. brucei* provides the positional cues to cytokinesis (22). The FAZ joins the tip of a flagellum to the cell cortex. In a mature and nondividing cell, there is most likely a FAZ localized at the anterior tip of the cell. The cleavage furrow begins at the anterior tip and ingresses toward the posterior end and eventually dissects the cells (44). The identities of the FAZ and cleavage furrow remain unknown primarily due to the lack of an identifiable protein marker for either of them (36). Our current study, indicating that TbPLK is likely localized in the FAZ in tight association with the four specialized microtubules, could be an important discovery. By indicating the likely localization of TbPLK in the FAZ, it enabled us to visualize the potential presence of multiple FAZs and localize them in a chain of well-defined punctate distribution beneath the plasma membrane where the undulated membrane is known to extend out to wrap around the flagellum. The very last FAZ in the chain at the anterior tip of the cell is apparently more exposed, because the TbPLK in it could be immunostained without prior detergent treatment. It also contains the highest quantity of TbPLK, which raises the interesting possibility that the kinase activity in this particular FAZ may have the function of triggering cytokinesis and cell division. When the daughter flagellum grows to its full length in a cell ready to divide, two exposed and concentrated stores of TbPLK come together at the anterior tip of the cell. We postulate that it is that very event that enables TbPLK to trigger cytokinesis and cell divi-



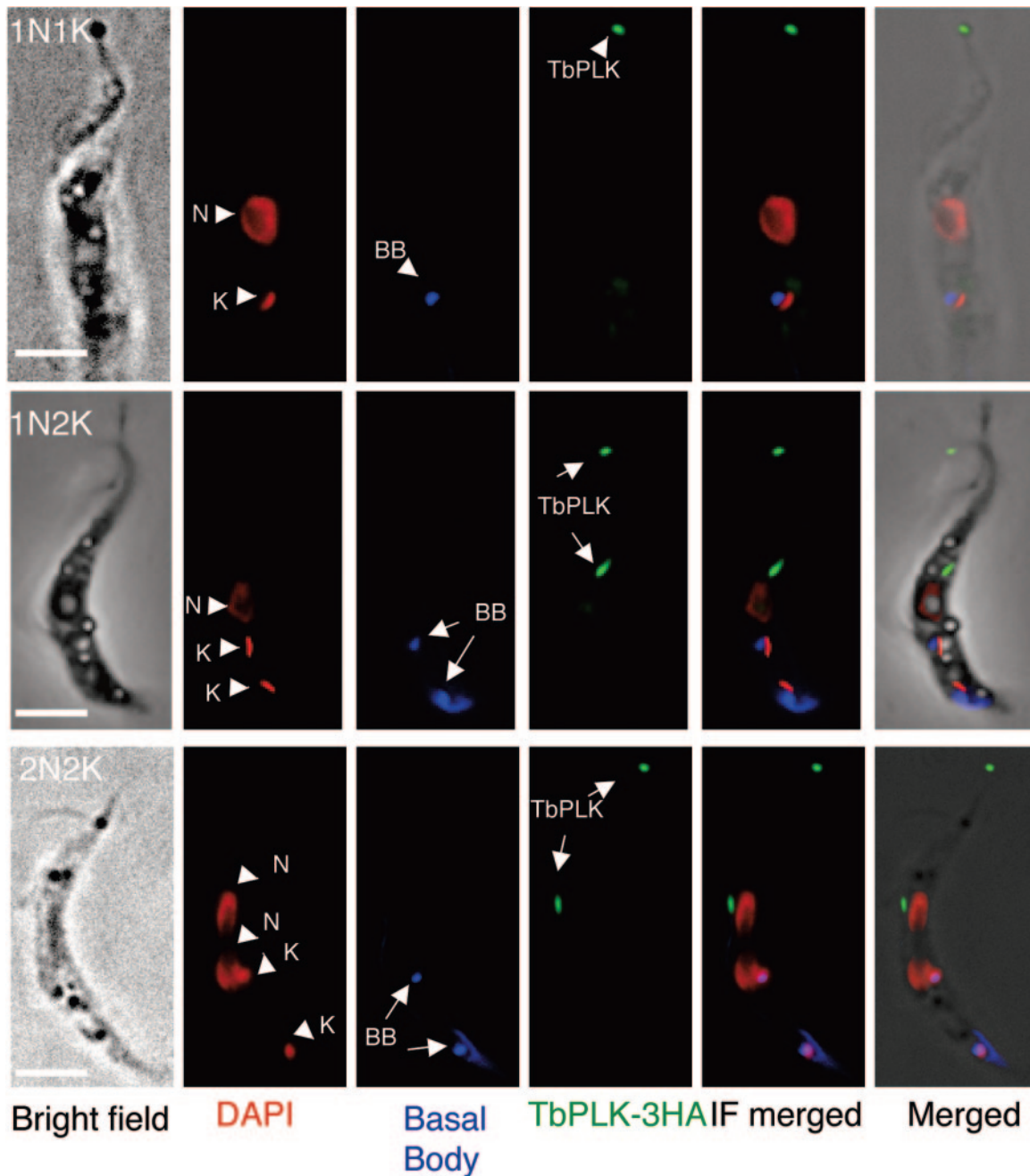


FIG. 8. Localization of TbPLK in procyclic-form *T. brucei*. Cells transfected with the overexpression vector containing TbPLK-3HA were treated with tetracycline (0.1  $\mu\text{g}/\text{ml}$ ) for 5 days. The cells were harvested, fixed, and used to coat poly-L-lysine coverslips for immunostaining. Immunostaining of basal bodies (BB) is in blue, and that of TbPLK-3HA is in green. Nuclei (N) were stained with DAPI 9 (red). Images were first processed with Imagej 1.33u (NIH) and then modified with Adobe Photoshop CS. K, kinetoplast; IF, immunofluorescence images. Bars, 5  $\mu\text{m}$ .

sion. Since flagellum growth is initiated by kinetoplast segregation and basal body duplication, it is little wonder that cytokinesis appears to be primarily controlled by the kinetoplast cycle (23). However, in the absence of TbPLK, cytokinesis cannot be initiated even after the daughter flagella are fully grown. This also points out the fact that the presence of TbPLK in the FAZ is not required for flagellum growth, since multiple full-length flagella were observed in TbPLK knock-down cells.

The presence of a chain of apparent FAZs along the dorsal side of a nondividing cell and the growth of a second line of apparent FAZs that closely parallels the existing one in a dividing cell are biological phenomena never observed or reported for another living organism, to our knowledge. These two parallel lines of potential FAZs are positioned at the presumed cleavage furrow where longitudinal cleavage takes place during cell division. It is thus tempting to postulate that the two lines of apparent FAZs may be closely associated with

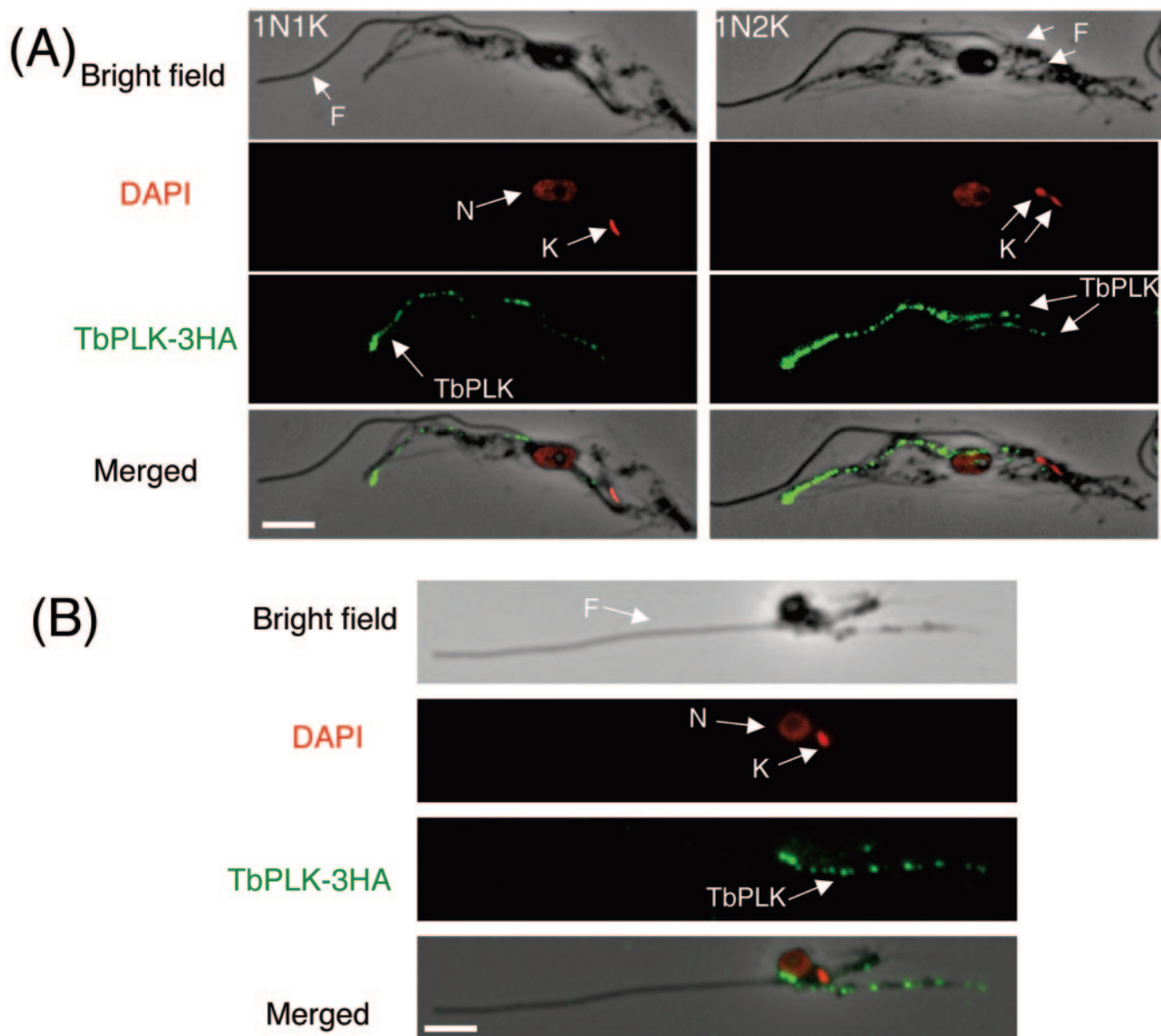


FIG. 9. TbPLK is apparently localized in the FAZ. The cytoskeleton and flagella were prepared from TbPLK-3HA-expressing procyclic-form *T. brucei* cells and underwent immunofluorescence assays. (A) Immunolocalization of TbPLK-3HA in isolated cytoskeletons. (B) Immunolocalization of TbPLK-3HA in high-salt-treated cytoskeletons. F, flagellum; N, nucleus; K, kinetoplast. Bars, 5  $\mu$ m.

the cleavage furrow or even constitute a part of it in trypanosomes. A probable triggering of cell division at the anterior tip of the cell by joining the two TbPLK reservoirs together could be followed by similar events for the rest of the TbPLK stores down the two parallel lines toward the posterior end. It could be a zipperlike cascade that accomplishes the task of trypanosome cell division.

In conclusion, we have identified TbPLK as the initiating factor for cytokinesis in the procyclic form of *T. brucei*. Though TbPLK can complement the functions of Cdc5, it apparently does not play a significant role in the  $G_2/M$  transition, the metaphase-anaphase transition, anaphase release, or mitotic exit. Nor is TbPLK involved in regulating kinetoplast segregation. Its probable localization in a punctate line of likely FAZs

in nondividing cells and the formation of two such lines in parallel in dividing cells suggest the interesting possibility that TbPLK is localized in the cleavage furrow and performs a crucial function for cell division. This is a well-defined, unique, and interesting model for studying cytokinesis and cell division. It may provide an opportunity for further in-depth analyses of cytokinesis and cell division regulation independent of mitosis and all the other events occurring during the cell cycle.

**ACKNOWLEDGMENTS**

We thank Zacheus Cande of UC Berkeley for the many stimulating and inspiring discussions we had with him throughout this project and for his critical reading of the manuscript. Gratitude also goes to Keith Gull of Oxford University for the kind gifts of antibodies L8C4 and

KMX-1, to Kevan Shokat of UCSF for providing the yeast strains, and to Jack Taunton of UCSF for the use of the fluorescence microscope. This work was supported by NIH R01 grant AI-21786.

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