Polo-Like Kinase Guides Cytokinesis in *Trypanosoma brucei* through an Indirect Means*<sup>*</sup>  

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Received 6 November 2009/Accepted 1 March 2010

Polo-like kinase in *Trypanosoma brucei* (TbPLK) is confined to the flagellum attachment zone (FAZ) and regulates only cytokinetic initiation. However, it apparently diffuses into the cytoplasm before the trans-localization of chromosomal passenger complex (CPC) from the midzone of central spindle to FAZ, which is known to be required for initiating cytokinesis. Synchronized *T. brucei* procyclic cells treated with a TbPLK inhibitor, GW843682X (GW), in late S phase were found to go through a full cell cycle at a normal pace before being arrested at cytokinetic initiation in the second cycle. However, synchronized cells treated with GW in G<sub>1</sub> phase were arrested at cytokinetic initiation within the first cell cycle, suggesting that inhibition of TbPLK at its emergence blocks cytokinesis within the same cell cycle. To rule out potential off-target effects from GW, TbPLK RNA interference (RNAi) was induced to deplete TbPLK, and the progression of synchronized cells from late S phase was also found to be arrested at cytokinetic initiation within the first cell cycle. Apparently, TbPLK has accomplished its role in guiding cytokinesis before the late S phase, presumably by phosphorylating a certain substrate(s) during S phase, which may play a critical role in initiating the subsequent cytokinesis.

Cell cycle progression in eukaryotes is tightly regulated, requiring participation of many regulatory proteins. The surveillance mechanisms, controlled under various checkpoints, monitor the integrity of cell cycle progression and ensure that genetic information is correctly transmitted to daughter cells (11, 19). Aurora kinase B and Polo-like kinase 1 (PLK1) are among the major protein kinases playing multiple roles in regulating both mitosis and cytokinesis. Aurora B is known to regulate chromosomal segregation as well as cytokinetic initiation (38). It forms a chromosomal passenger complex (CPC) with inner centromere protein (INCENP), Survivin, and Borealin/Dasra (10). CPC binds to the kinetochores. It detects and corrects aberrant kinetochore-microtubule attachments by phosphorylating several key kinetochore-centromere components (42), activating the spindle checkpoint (43), and inhibiting the anaphase-promoting complex/cyclosome (APC/C) to arrest cells in metaphase (29, 34). Once the kinetochore-microtubule attachments have been perfected, the spindle checkpoint becomes inactivated and the cell cycle proceeds to anaphase. An Aurora B-mediated phosphorylation of the subunits in the centraspinidlin complex then targets centraspinidlin and CPC to the central spindle midzone, where Ect2-bound centraspinidlin activates the small GTPase RhoA to promote formation of the actomyosin contractile ring that constitutes the initial cleavage furrow (15, 28, 33, 46). The ring then closes onto the midzone to complete the process of cytokinesis (3).

PLK1 also plays multiple roles in regulating G<sub>2</sub>/M transition (1, 37), metaphase/anaphase transition (2), anaphase release (20), mitotic exit (18), and initiation of cytokinesis (6, 14, 45) in yeasts and metazoans. It localizes in the nucleus throughout the cell cycle, during which expression of a particular function of PLK1 depends on its subcellular localization (30, 31), whereas the particular localization of PLK1 hinges on binding, through its two Polo boxes, to specific targets available at a given time. PLK1 is targeted to the centrosome and kinetochores in early mitosis by binding to phosphorylated centrosomal CDC25C phosphatase (7, 22) and phosphorylated kinetochore-associated Bub1, respectively (17, 36). A dramatic relocation of PLK1 from the centrosome and kinetochores to the spindle midzone during the subsequent metaphase-anaphase transition is accomplished by an association of PLK1 with PRC1 and MKLP2; both are phosphorylated by PLK1 to become a target for the Polo boxes in PLK1, which in turn relocalize PLK1 to the spindle midzone (30, 45). Once localized there, PLK1 recruits RhoGEF and Ect2, which then attracts centraspinidlin, which is associated with CPC to initiate cytokinesis (3, 6, 15, 28, 33, 35, 45, 46). Thus, PLK1 and CPC form a complex in the spindle midzone during late anaphase for cytokinetic initiation.

*Trypanosoma brucei* is a deeply branched protozoan parasite which causes African sleeping sickness in humans and nagana in various livestock. It divides longitudinally from the anterior toward the posterior end of cell in a pattern totally different from that of metazoans and yeasts. There is a single Aurora-like kinase homologue, TbAUK1, in *T. brucei*, which is responsible for promoting spindle assembly and chromosome segregation, as well as cytokinesis (39). Homologues of INCENP, Borealin, and Survivin have not been found in the *trypanosome* genome (5). Instead, a CPC consisting of TbAUK1 and...
two novel proteins, TbCPC1 and TbCPC2, bearing no structural similarity to those three proteins was identified in *T. brucei* (27). This CPC in *T. brucei* displays a subcellular trans-localization pattern similar to that of the metazoan CPC during mitosis (41) by associating with chromosomes during G2 phase, binding to the apparent kinetochores in metaphase, and then moving to the central spindle midzone in anaphase. It then further trans-localizes in a pattern that has never been observed in other eukaryotes. The central spindle bends toward the dorsal side of the cell, where the flagellum attachment zone (FAZ) is located, in late anaphase. CPC in the midzone is then transferred, apparently across the nuclear envelope, to the midpoint of FAZ and then moves to the anterior end of cell to initiate cytokinesis by moving toward the posterior end to divide the cell (27). This highly unusual mode of cell division in *T. brucei* indicates a fundamentally different mechanism of cytokinesis that could apply to flagellated eukaryotes that divide longitudinally.

There is also only a single PLK homologue, TbPLK, in *T. brucei* (13). It is capable of complementing deletion of the PLK Cdc5 from *Saccharomyces cerevisiae*, suggesting that it possesses all the functions of Cdc5 (23). However, TbPLK controls only cytokinesis in *T. brucei*. RNA interference (RNAi) of TbPLK produces cells with multiple nuclei, kinetoplasts, basal bodies, and flagella (23). TbPLK does not localize to the nucleus but is associated with the posterior end of FAZ when it emerges during S phase and then moves along the growing tip of the new FAZ toward the anterior end (23, 40). Since an interaction of PLK and Aurora B in the midzone has been described (26). Briefly, synchronized cell cycle progression from G1 phase. The procyclic forms of *Trypanosome* cell culture.

Synchronization of the procyclic form of *T. brucei*. For synchronization of cells to the early S phase, cells expressing tagged TbAUK1-EYFP, TbCPC1-EYFP, and TbCPC2-EYFP were treated with 0.3 mM hydroxyurea for 16 h (8). The hydroxyurea was then washed off with fresh medium, and the cells were incubated in fresh medium for synchronous cell cycle progression. GW (5 μM) in DMSO was added to the synchronized cells at 0 h after the release.

For synchronization of cells in the G1 phase, cells expressing TbAUK1-EYFP, TbCPC1-EYFP, or TbCPC2-EYFP were cultivated in *vitro* at 26°C for 4 days until the cells reached the stationary phase with a cell density of 1 × 10^7/ml (see Results). The cells were then resuspended in fresh medium and released for synchronized cell cycle progression from G1 phase. For synchronization of cells harboring the TbPLK-RNAi vector and expressing endogenously tagged TbAUK1-EYFP, TbCPC1-EYFP, or TbCPC2-EYFP were cultured in *vitro* at 26°C for 4 days until the cells reached the stationary phase with a cell density of 1 × 10^7/ml (see Results). The cells were then resuspended in fresh medium and released for synchronized cell cycle progression from G1 phase.

Flow cytometry analysis. Fluorescence-activated cell sorter (FACS) analysis of propidium iodide (PI)-stained trypanosome cells was carried out as previously described (26). Briefly, *T. brucei* cells were spun down at 320 × g for 10 min, washed once in phosphate-buffered saline (PBS), and resuspended in 0.1 ml PBS. The cells were fixed by adding 0.2 ml of 10% formalin, 0.2 ml of 50% ethanol, and 1.0 ml of 70% ethanol (all in PBS with 5% glycerol) and incubated at 4°C. They were then spun down again at 2,900 × g for 10 min, washed once with PBS, and resuspended in PBS. DNase-free RNase (10 μg/ml) was added before the flow cytometry analysis. The DNA content of PI-stained cells was analyzed with a
in anaphase before the trans-localization of TbAUK1 from the midzone to the midpoint of FAZ in late anaphase. The apparent diffusion of TbPLK from FAZ into the cytoplasm during anaphase could be attributed to either a loss of TbPLK function or a dissociation of the anchor of TbPLK from FAZ (see Discussion). Thus, there is probably no complex formation between TbPLK and CPC on FAZ during late anaphase.

The PLK1 inhibitor GW inhibits TbPLK activity and blocks growth of T. brucei procyclic cells at cytokinetic initiation. A selective thiophene benzimidazole ATP-competitive inhibitor of PLK1 and PLK3, 5-(5,6-dimethoxy-1H-benzimidazol-1-yl)-3-[2-(trifluoromethyl)-benzoyl]-thiophene-2-carboxamide (GW843682X [GW]), has recently become commercially available (Tocris Cookson Inc.). It inhibits PLK1 and PLK3 with 50% inhibitory concentrations (IC50s) of 2.2 and 9.1 nM, respectively, in an enzyme assay that, in our opinion, might have overestimated the potency of GW (24) (see below). It inhibits the proliferation of a wide variety of tumor cell lines with much higher IC50s, ranging from 0.38 μM on human lung adenocarcinoma cells to 6.82 μM on human diploid fibroblast cells (24). When tested at 1 to 3 μM on the human lung adenocarcinoma cell line, GW reduced G1 and S phases and induced a transient G2/M arrest, but it significantly enhanced the population of sub-2N and >4N cells after 72 h. When GW was used to treat human diploid fibroblast cells in the same concentration range, G1 cells were reduced while G2/M cells were enriched, with a small increase in multinucleate cells, after 72 h. These phenotypes reflected inhibition of the multiple functions of PLKs in mammalian cells and suggested a specific action of GW on the PLKs in vivo under the experimental conditions used (24).

To examine whether GW can also inhibit TbPLK, Escherichia coli-expressed recombinant glutathione S-transferase (GST)-tagged TbPLK was purified and used in an in vitro kinase assay with [γ-32P]ATP and dephosphorylated casein as substrates in the presence of various concentrations of the drug. TbPLK phosphorylated casein strongly in the no-drug control but was inhibited by increasing drug concentrations, with an estimated GW IC50 of 1.3 μM (Fig. 2A). The potential effect of GW on the growth of T. brucei procyclic cells was tested with increasing concentrations of GW from 1 to 5 μM (Fig. 2B). Cells grown with 1 μM GW grew at a slightly lower rate than the no-drug control cells. The growth rate became significantly reduced in the presence of 2 to 3 μM GW, and growth was virtually stopped by 4 to 5 μM GW, which led eventually to cell death after 4 days of incubation. The IC50 of GW for cell growth was estimated to be 2 μM, which is in good agreement with the IC50 of 1.3 μM against TbPLK from our enzyme assay. The much lower IC50s of GW against mammalian PLKs than that reported against TbPLK could be attributed to different enzyme assaying methods. The one used for the mammalian enzymes has the enzyme protein deleted of its multiple functions, while the other used for TbPLK includes the full-length protein. This may account for the difference.

RESULTS

**TbPLK trans-localizes from FAZ to cytoplasm during anaphase.** T. brucei procyclic cells expressing both TbAUK1-EYFP and TbPLK-mCherry through homologous genetic recombination were synchronized with 0.3 mM hydroxyurea to late S phase and released (8). Cell samples were taken hourly and examined with a fluorescence microscope. The results (Fig. 1) indicate that during the initial 2 h, TbAUK1 became concentrated from a spread throughout the nucleus onto the metaphase plate, reflecting a cell cycle progression from S phase to metaphase. Meanwhile, TbPLK apparently moved from the posterior end to the midpoint of FAZ (40). Between the third and fourth hours after the release, TbAUK1 moved to the midzone of the central spindle, signaling anaphase. However, the focal point of TbPLK-mCherry on the cellular dorsal side became faded and apparently diffused into the cytoplasm after 4 h, suggesting vanishing of TbPLK from FAZ.
To further examine the effect of GW on cell cycle progression, samples of unsynchronized procyclic cells incubated with different concentrations of GW for different times were stained with propidium iodide (PI) for DNA and analyzed by flow cytometry (Fig. 2C). After 1 day of drug treatment, there was an enrichment of G2/M-phase cells (4C DNA content), which increased with higher concentrations of GW, accompanied by a corresponding decrease of G1-phase cells (2C DNA content). At the highest concentration of GW (5 μM), there were signs of emergence of cells with 8C DNA content within 1 day, indicating that the cells failed to divide but instead went through another mitotic cycle to produce four nuclei in the cell. This result is in agreement with that obtained after knocking down TbPLK from the procyclic cells by RNAi (23), except that it took longer (2 days) for RNAi to show the appearance of 8C cells, which could be attributed to likely lower turnover rates of TbPLK mRNA and protein. When the incubation time was lengthened to 2 or 3 days, the drug effect became even more pronounced, with a steady increase of the 8C cells with increasing drug concentrations. Thus, GW apparently has an effect similar to that of TbPLK RNAi on cell cycle progression, indicating a specific inhibitory effect of GW on TbPLK under the present experimental conditions. This also confirms our previous observation from the RNAi study (23) that TbPLK regulates only cytokinesis in T. brucei.

GW treatment of late-S-phase cells has no immediate effect on progression of the cell cycle but blocks cytokinetic initiation in the second cycle. Strain 427 procyclic cells expressing CPC1-EYFP through homologous genetic recombination were synchronized with 0.3 mM hydroxyurea to late S phase and released. GW (5 mM) was added at time zero of the release, and cell cycle progression was monitored hourly thereafter by flow cytometry. A more detailed examination was also conducted simultaneously by following the pattern of CPC1-EYFP trans-localization with a fluorescence microscope, serving as another, more detailed indicator of cell cycle progression.

Data from flow cytometry of the no-drug control showed that the synchronized cells started mostly from late S phase (with a small percentage of G1 cells still present) and proceeded quickly into the G2/M phase within the first hour (Fig. 3A). G1 cells, representing the daughter cells from cell division, then began to emerge and reached the highest level between the fourth and fifth hours. S-phase cells then began to reappear, reaching a profile by the eighth hour similar to that observed at 0 h, thus completing a well-synchronized cell cycle within 8 h.

When GW was added to the late-S-phase cells at time zero, the cells progressed almost exactly like those in the no-drug control during the initial 8 h. Essentially all the cells divided and returned to S phase again to complete the first synchronized cell cycle (Fig. 3A). Thus, the drug exerted no detectable effect on progression of the first cell cycle. The cells then progressed into the G2/M phase of the second cell cycle at the
ninth hour, but no further conversion of G2/M cells to G1 cells could be observed with GW thereafter. The G2/M cells kept accumulating up to the 12th hour until essentially most of the cells were in the G2/M phase. A C8 cell population then began to emerge (Fig. 3A). This unusual observation, further quantitatively analyzed in Fig. 3B, indicated that inhibition of the TbPLK function in late S phase does not have an effect on the progression of the first cell cycle but stops cytokinesis in the second cycle. Thus, the TbPLK function expressed during S phase prior to the addition of GW in the late S phase is apparently of pivotal importance in guiding the occurrence of cytokinesis within the same cell cycle.

In the cells released in late S phase, trans-localization of TbCPC1-EYFP during the cell cycle progression was also monitored hourly with a fluorescence microscope for more detailed information. Approximately 200 cells in each sample were examined and categorized as interphase, metaphase, anaphase, late anaphase, “CPC1 on bent midzone,” or “CPC1 on anterior tip” by localizing TbCPC1-EYFP and comparing it with the DAPI stain. In the no-drug control, TbCPC1-EYFP was identified mostly in a punctate distribution in the nucleus at the beginning (0 h), representing the interphase (Fig. 4A). The population of interphase cells dropped quickly within the first 4 h but climbed back to a peak within 8 to 9 h, corresponding roughly to the time period of one cell generation. The metaphase cells also reached peak levels twice, at the 2nd and 10th hours. The anaphase and late-anaphase cells and the cells with TbCPC1-EYFP moving from the bent midzone to the anterior tip all emerged twice and peaked at the 3rd and 11th hours, respectively (Fig. 4A). Thus, within 12 h, the synchronized control cells progressed from late S phase through one full cell cycle and reached cytokinetic initiation in the second cell cycle.

When GW was added to the synchronized late-S-phase cells at time zero, no apparent change from the control was observed in the first cell cycle progression until no TbCPC1-EYFP was detectable on the anterior tip in the second cell cycle.
cycle at the 11th hour (Fig. 4B). Cytokinetic initiation was blocked at this specific TbCPC1-EYFP trans-localization in the second cycle. This delayed response to inhibition of TbPLK in the late S phase until the next cell cycle suggests that the enzyme performs and completes its crucial function in directing cytokinetic initiation upon its first appearance in the S phase when GW is not yet added. The lack of any detectable effect of 5 μM GW on progression of the entire first cell cycle also strongly suggests an absence of any off-target effect of GW on T. brucei cell cycle progression.

Similar experiments monitoring the effects of GW on trans-localization of the other two tagged CPC subunits, TbAUK1-EYFP and TbCPC2-EYFP (data not shown), during cell cycle progression were also performed. The results were essentially the same as those shown in Fig. 4.

This unusual phenomenon could have a relatively simple explanation. When TbPLK emerges in S phase, it may start to phosphorylate a certain substrate(s). Thus, when GW is added in late S phase, the substrate(s) has already been phosphorylated and becomes capable of promoting cytokinetic initiation within the same cell cycle, even though TbPLK becomes inhibited by GW in the late S phase. If this hypothesis is true, then GW added to the G1-phase cells prior to the emergence of TbPLK is expected to inhibit cytokinetic initiation within the first cell cycle.

GW treatment of G1-phase cells blocks cytokinetic initiation within the first cell cycle. Despite a few reports in meeting abstracts describing synchronization of T. brucei procyclic cells to the G1 phase by nutrient starvation, there has not yet been a method published in the literature. We thus followed the published method for synchronizing the budding yeast S. cerevisiae to G1 phase through nutrient starvation (21). T. brucei strain 427 procyclic cells expressing TbCPC1-EYFP were inoculated into fresh culture medium (see Materials and Methods) to an initial density of 1 × 10^5 cells/ml and incubated at 26°C for 4 days. The cells reached stationary phase with a density of 1 × 10^7 cells/ml. Flow cytometry analysis indicated 95% G1 cells, 3% S-phase cells, and virtually no detectable G2/M cells in the population (Fig. 5B, time zero). The cells were harvested and released in fresh medium. During the initial 10 h, there was a slow but steady decrease of G1-phase cells accompanied by a corresponding increase of S-phase cells (Fig. 5B). Apparently, the cells had a much-reduced rate of crossing the G1/S boundary after long-term starvation, reflecting a need for recovery from many potential defects accumulated during nutrient starvation. However, from the 11th hour on, most of the cells were in S phase. G2/M cells began to emerge and increased in number, reaching the maximum level at the 15th hour. They then began to decrease, while G1 cells, derived from apparent cytokinesis of G2/M cells, started to rise.

FIG. 4. Effects of GW on trans-localization of TbCPC1-EYFP in T. brucei procyclic cells synchronized and released in the late S phase. Strain 427 cells expressing TbCPC1-EYFP at the apparent endogenous level were synchronized to the late S phase with hydroxyurea and released without (A) or with (B) 5 μM GW added at time zero. Cell samples were taken hourly, stained with DAPI, and examined with a fluorescence microscope. The nucleus (N) and kinetoplast (K) are shown in red, and TbCPC1-EYFP is shown in yellow. Approximately 200 cells were examined in each sample and classified into interphase, metaphase, anaphase, late anaphase, cells with CPC1 on the bent midzone, and cells with CPC1 on the anterior tip. Numbers of cells in each category were tabulated and presented in histograms. Error bars indicate standard deviations.
from the lowest level at the 13th hour to the highest at the 19th hour. The S-phase cells, decreasing between the 12th and 17th hours in coordination with the increasing G2/M population, began to rise again at the 17th hour. This well-synchronized cell cycle progression took about 8 h between the appearance of the first and second peaks of S phase, agreeing with the previously estimated generation time for *T. brucei* strain 427 procyclic cells (Fig. 3A). The starvation-synchronized cells had thus apparently regained the healthy state after a 10-hour recovery period in fresh medium, and the cell cycle proceeded at the regular pace in a synchronous manner thereafter.

When GW was added to the cells synchronized in G1 phase at time zero, the recovery of cells during the initial 10 h proceeded essentially identically to that in the no-drug control (Fig. 5A and B), suggesting a lack of any drug effect on the recovery phase. However, after this period, the G2/M cells started to increase steadily without any sign of stopping up to the 20th hour. G1 cells continued to decrease, whereas S-phase

FIG. 5. Effects of GW on progression of *T. brucei* procyclic cells synchronized and released in the G1 phase. (A) Procyclic strain 427 cells were synchronized to G1 phase after nutrient deprivation and released with 5 μM GW added at time zero. Cell samples were harvested hourly afterward and subject to flow cytometry analysis for DNA content. (B) Analysis of the data from flow cytometry with ModFit LT V3.0 software for estimation of percentages of cells in G1, S1, and G2/M DNA phases.
cells, after reaching the highest level at the 12th hour, also decreased steadily. Thus, a major difference between the drug-treated cells and the control cells was in the continued increase of G2/M cells accompanied by a steady decrease of G1- and S-phase cells, indicating an inability of G2/M cells to go through cytokinesis. Thus, one apparent difference between the effects of the drug on cells synchronized in late S phase and G1 phase is that it allows progression of the former through a full cell cycle prior to inhibition of cytokinesis in the second cell cycle, whereas cytokinesis in the latter becomes inhibited within the first cell cycle. GW may have accomplished something crucial by being present in the G1 phase instead of the late S phase, suggesting the importance of TbPLK function during its emergence in the S phase.

The pattern of trans-localization of TbCPC1-EYFP after releasing the G1-phase cells was also examined in the absence of GW (Fig. 6A). The interphase cells reached the maximum level after 12 h. They then started to decline but began to rise again at the 20th hour, resembling those synchronized in late S phase progressing between time zero and the 6th hour in the previous experiment (Fig. 5B). Cells in metaphase peaked after 13 h, whereas those in anaphase, those in late anaphase, those with CPC on the bent midzone, or those with CPC1 on the anterior tip appeared sequentially afterwards with each one in a single peak up to the 17th hour, when G2/M cells were decreasing while G1 cells were increasing (Fig. 5B).

When GW was added to the G1 cells at time zero and the localization of TbCPC1-EYFP was followed, the interphase cells first increased as in the control and then decreased between the 12th and 16th hour in a single peak without a subsequent increase toward the 20th hour (Fig. 6B). Metaphase cells kept increasing and reached the highest level at this hour. The anaphase cells remained relatively constant in number, while late-anaphase cells and cells with CPC1 on the bent midzone were also increasing, with the maximum levels seen at the 20th hour. There was, however, no cell with CPC1 located on the anterior tip detectable, suggesting that trans-localization of CPC1 from the bent midzone to the anterior tip was inhibited by GW. Cytokinetic initiation was thus inhibited in the first cell cycle when GW was added in G1 phase.

trans-Localizations of TbAUK1-EYFP and TbCPC2-EYFP (data not shown) in cells treated with GW from G1 phase onward were also examined, and the results were the same as those from the TbCPC1-EYFP experiments described above.

*T. brucei* procyclic cells depleted of TbPLK by RNAi and synchronized to late S phase have cytokinetic initiation blocked within the first cell cycle. To rule out the possibility of potential off-target effects from GW that could have caused
some of the phenotypes observed in the experiments described above, we depleted TbPLK by RNAi as described in our previous study (23). The cells are known to continue growing at the same rate as the wild type within the first 1 to 2 days after RNAi induction (23). We thus first induced the RNAi for 24 h and then treated the cells with 0.3 mM hydroxyurea for an additional 16 h to synchronize them to the late S phase. The TbPLK level was determined by Western blotting using a rabbit polyclonal antibody and analyzed by densitometer tracing. The results (Fig. 7) showed that TbPLK was decreased to 14% of the original level after 24 h of TbPLK RNAi induction and became undetectable after another 16 h of RNAi with hydroxyurea treatment. The cells had thus been deficient in TbPLK during the 16 h of hydroxyurea treatment before they were released in late S phase. The depletion of TbPLK from 14% to 0% during the second phase of the 16-hour RNAi and hydroxyurea treatment suggested that the kinase had been deficient for one cell generation or more before the release in late S phase, and the cells would be thus blocked at the cytokinetic initiation within the first cell cycle. The TbCPC1-EYFP-expressing and TbPLK-depleted cells, synchronized in late S phase, were released, and the subsequent cell cycle progression was monitored with flow cytometry. The RNAi-treated cells progressed similarly to the control cells in the initial 5 h, when late S-phase cells were transformed into G2/M-phase cells (Fig. 8A and B). Then, between the fifth and eighth hours, the TbPLK-depleted cells

FIG. 7. Depletion of TbPLK by RNAi. T. brucei procyclic strain 29-13 cells harboring the pZJM-TbPLK RNAi construct were treated with 1.0 μg/ml tetracycline for 24 h to induce TbPLK RNAi. While the RNAi was continuing, the cells were further treated with 0.3 mM hydroxyurea for 16 h to become synchronized to the late S phase. Cell samples prior to RNAi induction (0 h), after the 24-h induction (24 h), and after the hydroxyurea treatment (40 h) were lysed, subjected to SDS-PAGE, and analyzed by Western blotting using a rabbit polyclonal antibody to TbPLK obtained from Graham Warren. Intensities of bands were estimated by densitometer tracing. T. brucei α-tubulin was stained as a sampling control. 

FIG. 8. Effects of TbPLK depletion on the progression of T. brucei procyclic cells synchronized and released in late S phase. (A) Strain 29-13 cells harboring both pZJM-TbPLK RNAi and TbCPC1-EYFP constructs were treated with 1.0 μg/ml tetracycline for 24 h to induce TbPLK-RNAi. While the RNAi was continuing, the cells were further treated with 0.3 mM hydroxyurea (HU) for 16 h to become synchronized to the late S phase and released with continued TbPLK RNAi. Cell samples were collected hourly after the release and subjected to flow cytometry analysis for DNA content. (B) Analysis of the data from flow cytometry with ModFit LT V3.0 software for estimation of percentages of cells in G1, S1, and G2/M DNA phases.
became arrested in G₂/M phase without any sign of further progression to G₁ phase, while a population of cells with 8C DNA content began to emerge (Fig. 8A and B). Compared with the control cells, which showed active conversion from G₂/M to G₁ phase during the same period of time without any sign of 8C cells, the TbPLK-depleted cells were apparently blocked from cytokinesis within the first cell cycle similarly to GW-treated G₁ cells.

The trans-localization of TbCPC1-EYFP during this period was also monitored with a fluorescence microscope for 8 h. The control cells progressed from the late S phase to cytokinesis within this time span, with a continuous decrease in interphase cells and modest increases in metaphase, anaphase, and late anaphase cells as well as the cells with CPC1 on the bent midzone and cells with CPC1 on the anterior tip. For the TbPLK-depleted cells, there was a similar decrease in interphase cells and small increases in the other types of cells except for those with CPC1 at the anterior tip, which were hardly detectable, suggesting that trans-localization of CPC1 from the bent midzone to FAZ was blocked by TbPLK depletion (Fig. 9A and B).

Similar results were also obtained with the cells expressing EYFP-tagged TbAUK1 or TbCPC2 (data not shown). TbPLK depletion thus abolished the trans-localization of all three CPC subunits to the anterior tip within the first cell cycle, agreeing with our previous data from GW treatment of G₁-phase cells.

### DISCUSSION

In the present study, we have further consolidated our previous observation that TbPLK has unusual properties in comparison to the PLK1s in other eukaryotes (4, 32). It is localized in FAZ, and it is involved only in regulating cytokinesis in trypanosomes. Since TbPLK is capable of complementing an *S. cerevisiae* mutant depleted of Cdc5 (23), it apparently has all the necessary functions of Cdc5. Apparently, all it requires are the appropriate substrates or binding partners that emerge at specific times and specific locations during cell cycle progression. Thus, its local confinement in FAZ could cause its functional restriction in *T. brucei*, and its localization in FAZ could be attributed to the particular working environment inside a *T. brucei* cell. There has been no structural homologue of CDC25C, Bub1, PRC1, or MKLP2 found in the *T. brucei* genome (5), which may explain why TbPLK does not localize to the centrosome, kinetochores, or spindle midzone and is thus not involved in regulating centrosome maturation or metaphase-anaphase transition in *T. brucei*. The localization of TbPLK to FAZ could be attributed to the probable presence.

![Fig. 9. Effects of TbPLK depletion on trans-localization of TbCPC1-EYFP in *T. brucei* procyclic cells synchronized and released in the late S phase. Cell samples without (A) or with (B) TbPLK RNAi synchronized and released in late S phase were harvested every hour, stained with DAPI, and examined with a microscope.](http://ec.asm.org/)

*FIG. 9.* Effects of TbPLK depletion on trans-localization of TbCPC1-EYFP in *T. brucei* procyclic cells synchronized and released in the late S phase. Cell samples without (A) or with (B) TbPLK RNAi synchronized and released in late S phase were harvested every hour, stained with DAPI, and examined with a microscope. The nucleus (N) and kinetoplast (K) are shown in red, and TbCPC1-EYFP is shown in yellow. Approximately 200 cells were examined in each sample and classified into interphase, metaphase, anaphase, late anaphase, cells with CPC1 on the bent midzone, and cells with CPC1 on the anterior tip. Numbers of cells in each category were tabulated and presented in histograms. Error bars indicate standard deviations.
of a substrate(s) in FAZ that may bind to the two polo boxes of TbPLK and have TbPLK localized to FAZ. Thus, the local confinement in FAZ could restrict its function to only regulation of cytokinesis in T. brucei.

The involvement of TbPLK in regulating cytokinesis in T. brucei and its localization to FAZ prompted us to first look into the possibility of formation of a complex between TbPLK and TbCPC in FAZ, because TbCPC is known to trans-localize to the midpoint of FAZ to initiate cytokinesis (27). The observation made in the present study that TbPLK was apparently diffused from FAZ into the cytoplasm before TbCPC was to trans-localize from the central spindle midzone to the midpoint of FAZ suggests that they do not come together in FAZ prior to cytokinetic initiation. It is not known whether TbPLK diffused into the cytoplasm still retains its activity. Even if it does, it is unlikely that it could exert a regulatory function from the cytoplasm in directing the movement of TbCPC in FAZ to initiate cytokinesis. Thus, the regulation of cytokinesis by TbPLK (40) is most likely a function already accomplished before its dissociation from FAZ (see below). The subsequent unique movement of TbCPC in FAZ, first to the anterior end and then toward the posterior end of FAZ to divide the cell into two (27), is apparently accomplished without the presence of TbPLK. Furthermore, since structural homologues of Ect2, centralspindlin, or the actomyosin complex, which are known to play essential roles in cytokinesis in other eukaryotes, have also been found to be absent in T. brucei (5), the mechanism of cytokinesis in T. brucei could be highly distinctive from that in metazoans or yeasts and unique to the eukaryotes that divide longitudinally. It could be a completely different mechanism of cytokinesis representing a new research field for further exploration.

The disappearance of TbPLK from FAZ during anaphase, apparently by diffusion into the cytoplasm, raised the questions of how TbPLK becomes associated with the posterior end of FAZ upon its emergence in S phase at the beginning (40), how TbPLK becomes associated with the posterior end of FAZ, because TbCPC is known to trans-localize to the midpoint of FAZ to initiate cytokinesis (27). The observation made in the present study that TbPLK was apparently diffused from FAZ into the cytoplasm before TbCPC was to trans-localize from the central spindle midzone to the midpoint of FAZ suggests that they do not come together in FAZ prior to cytokinetic initiation. It is not known whether TbPLK diffused into the cytoplasm still retains its activity. Even if it does, it is unlikely that it could exert a regulatory function from the cytoplasm in directing the movement of TbCPC in FAZ to initiate cytokinesis. Thus, the regulation of cytokinesis by TbPLK (40) is most likely a function already accomplished before its dissociation from FAZ (see below). The subsequent unique movement of TbCPC in FAZ, first to the anterior end and then toward the posterior end of FAZ to divide the cell into two (27), is apparently accomplished without the presence of TbPLK. Furthermore, since structural homologues of Ect2, centralspindlin, or the actomyosin complex, which are known to play essential roles in cytokinesis in other eukaryotes, have also been found to be absent in T. brucei (5), the mechanism of cytokinesis in T. brucei could be highly distinctive from that in metazoans or yeasts and unique to the eukaryotes that divide longitudinally. It could be a completely different mechanism of cytokinesis representing a new research field for further exploration.

The disappearance of TbPLK from FAZ during anaphase, apparently by diffusion into the cytoplasm, raised the questions of how TbPLK becomes associated with the posterior end of FAZ upon its emergence in S phase at the beginning (40), how it migrates to the midpoint of FAZ in G2/M phase, and how it dissociates from FAZ in anaphase. From the accumulated knowledge on metazoans and yeasts, we could postulate that the two polo boxes in TbPLK may target the enzyme protein to the posterior end of FAZ, where a potential substrate of TbPLK may be localized. The subsequent movement of TbPLK along FAZ could be attributed to either migration of the bound target toward the midpoint or a continued change of targets by the enzyme toward the midpoint of FAZ. Eventually, the protein target in the middle portion of FAZ could become dissociated from FAZ and disassociate into the cytoplasm together with the TbPLK bound to it. Alternatively, TbPLK may become inactivated in anaphase, with its polo boxes incapable of binding to the target in FAZ. Some of the data from our preliminary study indicating a rapid disappearance of fluorescence-labeled TbPLK from FAZ upon addition of GW to the T. brucei cells (unpublished data) may lend some support to the speculation that TbPLK function is required for its localization to FAZ. These ideas could be readily tested by expressing a fluorescent-protein-tagged TbPLK mutant with the two polo boxes deleted. We are currently in the process of pursuing such a study.

Another question about the apparent dissociation of TbPLK from FAZ involves why it should vanish from FAZ at such a particular time and how it could regulate the initiation of cytokinesis while disappearing from FAZ ahead of the event. The use of an inhibitor of TbPLK, GW, and performance of TbPLK RNAi on T. brucei cells synchronized in the late S phase or G2 phase in the present study may have provided a partial explanation for this puzzle. Emerging during the S phase (40), TbPLK may have phosphorylated a certain substrate(s), whose phosphorylated form could be the actual regulator of cytokinetic initiation in T. brucei. TbPLK may diffuse from FAZ into the cytoplasm in anaphase, but the phosphorylated substrate(s) could remain localized to FAZ until late anaphase to direct the trans-localization of CPC from the midzone to FAZ or even to form a complex with CPC once the latter is trans-localized to FAZ for cytokinetic initiation. Thus, GW inhibition of TbPLK in late S phase would be too late to prevent phosphorylation of the substrate(s) at an earlier time. The latter will direct the progression of cytokinesis through the first cell cycle in a normal manner. When the newly divided cells have gone through G2 phase and the newly synthesized TbPLK is about to emerge in S phase, the continued presence of GW will inhibit any TbPLK activity from appearing in the second cell cycle. Consequently, the substrate(s) will not be phosphorylated in the second S phase, and cytokinesis in the second cell cycle will be inhibited. When T. brucei cells are treated with GW in G2 phase prior to the emergence of TbPLK or when TbPLK is depleted prior to the progression of cells into S phase, the substrate(s) would not be phosphorylated and cytokinesis would be inhibited within the first cell cycle. The outcome from our present study has fully verified these predictions. A positive identification of the substrate(s) should remain one of the most important goals for future investigation.

Few substrates of TbPLK have been identified in T. brucei thus far. TbPLK was found to emerge initially at the basal bodies and the posterior lobe of the bilobe structure near the Golgi apparatus (16). A Golgi apparatus-associated Ca2+-binding protein essential for Golgi duplication, TbCentrin2, was identified to be a substrate of TbPLK in vitro (9). Depletion of TbCentrin2 resulted in inhibition of T. brucei cell growth similar to that caused by TbPLK knockdown. However, TbCentrin2 is not associated with FAZ at any time during the cell cycle. Furthermore, depletion of TbCentrin2 inhibits Golgi apparatus duplication, whereas TbPLK deficiency results in multiple Golgi structures (16). Thus, if TbPLK phosphorylation of TbCentrin2 plays a role in regulating the subsequent cytokinetic initiation, involvement of other steps appears to be necessary. Future investigations will have to focus not only on identification of the substrate(s) of TbPLK but also on the potential involvement of additional events that may play pivotal roles in localizing TbPLK to FAZ and controlling cytokinesis in T. brucei.

ACKNOWLEDGMENTS

We thank Graham Warren of the University of Vienna for the rabbit polyclonal antibodies to TbPLK and George A. Cross of Rockefeller University for providing the T. brucei 427 and 29-13 strains. We are also grateful to Arthur Gunzl of the University of Connecticut Health Center for providing the pC-PTP-Neo vector and to Paul T. Englund of Johns Hopkins University School of Medicine for his gift of the pZJM plasmid. We also thank the Nikon Imaging Center at UCSF for...
the opportunity to use their 6D high-throughput microscope and Kurt Thorn for his technical assistance.

This work was supported by NIH R01 grant AI-21786.

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