Trypanosoma brucei FLA1 Is Required for Flagellum Attachment and Cytokinesis*

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The single flagellum of the protozoan parasite Trypanosoma brucei is attached along the length of the cell body by a complex structure that requires the FLA1 protein. We show here that inhibition of FLA1 expression by RNA interference in procyclic trypanosomes causes flagellar detachment and prevents cytokinesis. Despite being unable to divide, these cells undergo mitosis and develop a multinucleated phenotype. The Trypanosoma cruzi FLA1 homolog, GP72, is unable to complement either the flagellar detachment or cytokinesis defects in procyclic T. brucei that have been depleted of FLA1 by RNA interference. Instead, GP72 itself caused flagellar detachment when expressed in T. brucei. In contrast to T. brucei cells depleted of FLA1, procyclic T. brucei expressing GP72 continued to divide despite having detached flagella, demonstrating that flagellar attachment is not absolutely necessary for cytokinesis. We have also identified a FLA1-related gene (FLA2) whose sequence is similar but not identical to FLA1. Inhibition of FLA1 and FLA2 expression in bloodstream T. brucei caused flagellar detachment and blocked cytokinesis but did not inhibit mitosis. These experiments demonstrate that the FLA proteins are essential and suggest that in procyclic T. brucei, the FLA1 protein has separable functions in flagellar attachment and cytokinesis.

Trypanosoma brucei is an extracellular protozoan parasite that relies on a single flagellum for motility. This critical structure emerges from the flagellar pocket, a specialized secretory organelle near the posterior end of the cell, and extends along the cell body to the anterior tip. The flagellum contains an axoneme with the classical 9 + 2 bundle of microtubules and a paraflagellar rod (PFR) that is comprised primarily of two proteins, PFR-A and PFR-C (1, 2). The axoneme extends from the posterior end. Cross-links extend from the filament across the cell and flagellum membranes and into the PFR.

flagellum exits the flagellar pocket to the tip. The PFR is required for motility; inhibition of PFR-A expression by RNA interference (RNAi) ablates the PFR and paralyzes procyclic trypanosomes (3).

The flagellum is attached to the cell body via the flagellar attachment zone (FAZ), a complex but largely uncharacterized structure (4, 5). The FAZ is made up of an electron-dense cytoplasmic filament and a specialized set of four microtubules that are associated with the smooth endoplasmic reticulum (for a recent review of the T. brucei cytoskeleton, see Ref. 6). The filament is invariably located in a unique gap between two microtubules in the subpellicular cortex with the four microtubules always found immediately to the left when viewed from the posterior end. Cross-links extend from the filament across the cell and flagellum membranes and into the PFR.

During cell division, the flagellum and FAZ must be duplicated and segregated to the daughter cells. Synthesis of the new flagellum begins with duplication of the basal bodies at ~0.41 cell cycle units (5, 7). The new axoneme grows out from the basal body and emerges from the flagellar pocket. Axoneme emergence is followed by construction of the new FAZ beginning at about 0.52 cell cycle units (5, 7). Synthesis of the new FAZ begins before the construction of the new PFR; however, the PFR is then synthesized at a greater rate, and the formation of the new FAZ lags behind that of the new flagellum (8). Following mitosis and kinetoplast replication, a cleavage furrow that begins at the anterior tip and follows a helical path to the posterior end of the cell separates the daughter cells (5). In order for each cell to receive a flagellum and a FAZ, cleavage must occur between the old and the new FAZ. Given its invariant location and its unique link between the flagellum, basal bodies, and kinetoplast, the FAZ has been proposed to “mark the position and direction of the cleavage furrow” (9).

Although the identities of most components of the FAZ are unknown, at least one known T. brucei protein, flagellar adhesion glycoprotein 1 (FLA1), plays a critical role in flagellar attachment. FLA1 is a homolog of Trypanosoma cruzi GP72, an immunodominant protein localized to the junction between the T. cruzi flagella and the cell body (10–12). During the T. cruzi life cycle, GP72 is expressed primarily in the epimastigote (insect) stage and to a lesser extent in the metacyclic trypomastigote stage (11). Deletion of both copies of GP72 from the diploid T. cruzi genome yielded viable parasites with flagella that were detached from the cell body (13). The GP72 null mutants were immobile but divided at a normal rate in cell culture (13). However, the loss of GP72 dramatically reduced survival in the insect host (14).

FLA1 was identified in T. brucei as part of an expressed sequence tag sequencing project and is expressed in both the insect (procyclic) and mammalian (bloodstream) stages (15, 16). The 546-amino acid FLA1 protein is 44% identical and 63% similar to GP72 but lacks a threonine-proline rich region found
in the middle of the 581-amino acid GP72. FLA1 and GP72 have no significant homology to any other protein in the GenBank data base. Both FLA1 and GP72 have amino-terminal signal sequences that direct the proteins to the secretory pathway, a carboxyl-terminal transmembrane domain that anchors the proteins in the cell membrane, and a predicted 16-amino acid cytoplasmic tail (16). Similar to GP72, FLA1 is localized mainly to the region between the cell body and the flagella (16). In contrast to GP72, however, attempts to delete both copies of FLA1 from the T. brucei genome were unsuccessful (16), suggesting that FLA1 is essential in T. brucei.

We have used RNAi to transiently interfere with FLA1 expression in procyclic trypanosomes and have demonstrated that FLA1 is required for flagellar attachment to the cell body (17). These initial studies suggested that cells in which FLA1 expression was inhibited were unable to divide. To more fully evaluate the effect of the loss of FLA1, we have established permanently transfected procyclo and bloodstream cell lines that express FLA1 double-stranded RNA (dsRNA) upon induction with tetracycline. As expected, the loss of FLA1 was accompanied by flagellar detachment from the cell body. Cells expressing FLA1 dsRNA were unable to divide but continued to proceed through mitosis. Surprisingly, the expression of T. cruzi GP72 in procyclic T. brucei cells did not rescue either the flagellum detachment or the cytokinesis defect but instead itself caused flagellum detachment. Procyclic T. brucei expressing T. cruzi GP72 had detached flagella but were able to divide, suggesting that FLA1, but not necessarily flagellum attachment, is required for cytokinesis. We also identified an additional FLA1-like gene, called FLA2, whose sequence is similar enough to FLA1 to be inhibited by RNAi directed against FLA1.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—Plasmid p2TTT/AgFP was generated from plasmid pLEW82 (18) as follows. A SacII/HindIII fragment from pLEW82 was blunted with T4 DNA polymerase and inserted into SalI-digested, blunt-ended plasmid pLEW82 (plasmid A). The T7 RNA polymerase terminator sequences from pLEW82 were subcloned into the Psfl site of pBluescriptII SK (pBS). The resulting plasmid was digested with EcoRI and SalI, and the fragment containing the T7 terminator was ligated into the blunt SalI site from plasmid A (plasmid B). The GFP gene from pHDH-HX-GFP (19) was excised with HindIII and BamHI and ligated into the corresponding sites of pBS, giving pBS/GFP-H/B. The GFP gene and the multiple cloning sequence from pBS/GFP-H/B were excised with KpnI and SacI, blunt-ended with T4 DNA polymerase, and ligated into Smal-digested plasmid B (plasmid C). To fuse the T. brucei rRNA promoter to the BLE selectable marker gene, a KpnI/Smal fragment containing the RNA promoter from pH496 and a Smal/NcoI fragment from pLEW82 containing the act5' untranslated region and the 5' end of BLE was inserted into KpnI/NcoI-digested pBS/GFP-H/B via triple ligation (plasmid D). The 3' end of BLE and the 3' untranslated region were excised from pLEW82 with NotI and Psfl and cloned into the corresponding sites of plasmid D to regenerate a complete BLE gene (plasmid E). The RNA promoter-BLE construct from plasmid E was liberated with Psfl and KpnI, blunt-ended with T4 DNA polymerase, and ligated into the blunt-ended NheI site from plasmid C (plasmid F). To eliminate the NotI site introduced with GFP, plasmid F was partially digested with NotI, blunt-ended with T4 DNA polymerase, and religated, yielding p2TTT/AgFP.

To create p2TTT/BGFP, the DNA spacer region and T7 terminators from an isolate of plasmid B with the T7 terminators in the correct orientation was PCR-amplified using primers 5'-ATCCGGCGATCTCCTACAC-3' and 5'-CGAGAATTCGGACGCTATAGGTTG-3' and then cloned into pCR 2.1 using the Topo TA cloning kit (Invitrogen). After verifying by DNA sequence determination that the GFP gene contained no mutations, a HindIII/EcoRI fragment from plasmid I was ligated into the corresponding sites of pXSS2-pac (plasmid J). The KpnI/NotI fragment isolated from plasmid J was ligated into the corresponding site of plasmid p8k1, yielding p2TTT/BGFP.

Results

Construction and Characterization of Plasmid p2TTT—We have shown previously that two opposing bacteriophage T7 promoters can be used to generate RNAi in procyclic T. brucei expressing the T7 RNA polymerase (17). Using this system, we demonstrated that FLA1 is required for flagellar attachment in procyclic T. brucei. These experiments also suggested that cells with detached flagella are unable to divide, but we were unable to test this hypothesis because the original version of the
two-T7 promoter plasmid (p2T7) is not maintained episomally. Thus, to extend the length of time that RNAi can be maintained and to regulate the expression of RNAi, we developed an integratable version of the two-T7 promoter vector (p2T7Ti) that utilizes two tetracycline-inducible T7 promoters to generate sense and antisense RNA from the DNA sequences placed between them (Fig. 1).

Two versions of p2T7Ti are shown in Fig. 1 that differ only in the orientation of the second (rightmost) set of T7 transcription terminators (Fig. 1, the symbols /H9024/H9024). The T7 transcription terminators were included to prevent T7 RNA polymerase transcription from continuing through the adjacent rDNA. p2T7TiA and p2T7TiB yielded identical phenotypes for all genes tested. Black box, tetracycline operators; closed arrow, T7 promoter; open arrow, rRNA promoter; Ω, T7 transcription terminator.

To test the effectiveness of the p2T7Ti vectors, 500 bp from the 5’ end of the α-tubulin gene (TUB) were inserted between the two T7 promoters, and stable procyclic cell lines were established. Tetracycline was added to the culture media to induce dsRNA expression, and the cells were monitored for the appearance of the rounded FAT cell phenotype characteristic of inhibition of TUB expression (24). FAT cells appeared in 2T7TiA/TUB cultures within 6 h after adding tetracycline and reached a maximum by 18 h (Fig. 2). FAT cells were only rarely observed in control 2T7TiA/GFP cultures, indicating that the effect is due to inhibiting TUB expression. The percentage of FAT cells after adding tetracycline was much higher in cell lines cloned by serial dilution than in the uncloned mixed cell lines. Whereas uncloned mixed cell lines never had greater than 50% FAT cells after adding tetracycline, cloned lines were routinely obtained in which 95% of the cells become FAT after adding tetracycline. However, the high percentage of cells that displayed the FAT phenotype came at a price; lines with the highest percentage of FAT cells after tetracycline addition also showed evidence of leaky dsRNA expression. In uninduced...
cultures of these cell lines, 1–5% of the cells were FAT, and the cell lines grew more slowly than the 2T7TiA/GFP controls. Because inhibition of TUB expression is highly toxic, reversion to tetracycline-resistant phenotypes was observed. After 3 months of continuous passage in the presence of G418, hygromycin, and phleomycin, about half of the cells in the 2T7TiA/TUB line no longer responded to tetracycline addition. Furthermore, the tetracycline resistance was acquired much more rapidly when the cells were maintained in the absence of drug selection. The basis for resistance to tetracycline induction has not been analyzed further. For best results, 2T7TiA cell lines whose target genes are very toxic should be thawed and/or recloned every few months.

![Figure 2](image1.png)

**Fig. 2. Inhibition of TUB expression in procyclic p2T7/TiA/TUB cells causes the formation of FAT cells.** dsRNA expression was induced by adding tetracycline (Tet; 1 μg ml⁻¹) to procyclic cell lines containing integrated copies of p2T7/TiA/TUB (A and B) or p2T7/TiA/GFP (C and D). DIC images were obtained 12 h after adding tetracycline. Cell lines in the absence of tetracycline are shown for comparison (A and C). The scale bar indicates 10 μm.

Inhibition of FLA1 expression causes flagellar detachment in procyclic T. brucei—Having established that p2T7TiA efficiently generates RNAi in T. brucei in an inducible manner, we cloned 1000 bp from the 5’ end of FLA1 into p2T7TiA and obtained stable clonal lines. Procyclic cells with p2T7TiA/FLA1 integrated into the genome had normal morphologies in the absence of tetracycline but displayed detached flagella when grown in the presence of tetracycline (Fig. 3A). To verify that FLA1 expression was being inhibited, we analyzed FLA1 RNA levels in 2T7/TiA/GFP and 2T7TiA/FLA1 cells on northern blots (Fig. 3B). As expected, the ~3-kb FLA1 RNA disappeared in procyclic 2T7/TiA/FLA1 cells exposed to tetracycline. The loss of FLA1 RNA was accompanied by the appearance of a FLA1 RNA-related smear extending downward from ~1.5 kb. In contrast, neither FLA1 RNA levels in 2T7/TiA/GFP cells nor TUB levels in 2T7TiA/GFP or 2T7TiA/FLA1 cells were altered by tetracycline. Thus, as suggested previously by transient transfections (17), FLA1 is required for flagellar attachment in procyclic T. brucei.

**FLA1 Is Essential in Procyclic T. brucei**—The inability to delete both allelic copies of FLA1 from the diploid T. brucei genome (16) and our previous results with transient inhibition of FLA1 expression (17) suggested that FLA1 is essential in T. brucei. To test this hypothesis, we measured the growth rate of cell lines grown in the absence of tetracycline (Fig. 3A). As expected, the ~3-kb FLA1 RNA disappeared in procyclic 2T7/TiA/FLA1 cells exposed to tetracycline. The loss of FLA1 RNA was accompanied by the appearance of a FLA1 RNA-related smear extending downward from ~1.5 kb. In contrast, neither FLA1 RNA levels in 2T7/TiA/GFP cells nor TUB levels in 2T7TiA/GFP or 2T7TiA/FLA1 cells were altered by tetracycline. Thus, as suggested previously by transient transfections (17), FLA1 is required for flagellar attachment in procyclic T. brucei.

![Figure 3](image2.png)

**Fig. 3. Inhibition of FLA1 expression in procyclic T. brucei cells causes flagellar detachment.** dsRNA expression was induced in procyclic cell lines containing integrated copies of p2T7/TiA/FLA1 by adding tetracycline (Tet; 1 μg ml⁻¹) (A). DIC images were prepared 24 h after adding tetracycline. Cells in the absence of tetracycline are shown for comparison. The scale bar indicates 10 μm. FLA1 expression in procyclic 2T7/TiA/FLA1 and 2T7TiA/GFP was analyzed by northern blotting (B). Total RNA (5 μg) was isolated from the indicated cell lines grown in the absence or presence of tetracycline (1 μg ml⁻¹), subjected to gel electrophoresis, transferred to nylon, and probed sequentially with FLA1 and TUB. The ethidium bromide-stained gel is shown beneath the northern blots. Molecular size markers (in kb) are indicated.

absence or presence of tetracycline and at slightly greater rates than 2T7/TiA/FLA1 cells in the absence of tetracycline. These results demonstrate that FLA1 is essential for growth in T. brucei, in contrast to T. cruzi, where it has been shown that null mutants of the FLA1 homolog, GP72, can be obtained (13).

**T. brucei Cells Expressing FLA1 dsRNA Become Multinucleated**—While performing the growth curves shown in Fig. 4A, it became apparent that tetracycline-induced procyclic 2T7/TiA/FLA1 cells lose the normal trypanosome morphology, acquire a rounded phenotype reminiscent of FAT cells, and eventually die. A similar phenomenon was also observed with bloodstream 2T7/TiA/FLA1 cells induced with tetracycline (see below). To determine whether cells with detached flagella die at a particular stage in the cell cycle, we assessed the number of nuclei and kinetoplasts in 2T7/TiA/FLA1 cells grown in the presence or absence of tetracycline. During cell division in T. brucei, the kinetoplast replicates and segregates prior to nuclear segregation and thus can be used as a marker for progression through the cell cycle (5). Procyclic 2T7/TiA/FLA1 and 2T7/TiA/GFP cells were fixed with paraformaldehyde and stained with DAPI to visualize nuclear and kinetoplast DNA (Fig. 5). Control procyclic and bloodstream 2T7/TiA/GFP cells grown in the presence of tetracycline displayed the expected combinations of kineto-
plast and nuclei. Cells containing one nucleus and one kine
toplast (1N, 1K) predominated (Fig. 5, E and F) with fewer cells
having two kinetoplasts and one nucleus (2K, 1N) or two kin-
etoplasts and two nuclei (2K, 2N). In contrast, procyclic and
bloodstream 2T7TiA/FLA1 cells grown in the presence of tetracycline
developed multiple nuclei (>2N), sometimes exceeding
10 nuclei per cell (Fig. 5, A and B). The rounded morphology
noted above appears to develop because of the large number of
nuclei in the cell. Since in many cases the cells became packed
with nuclei, it was impossible to assess the exact number of
kinetoplasts present. Nonetheless, in at least some cells, three
or more kinetoplasts could be observed (Fig. 5, C and D). These
results indicate that although FLA1 is required for T. brucei
cytokinesis, FLA1 is not required for mitosis or kinetoplast
replication.

Expression of T. cruzi GP72 in T. brucei Causes Flagellum
Detachment—T. cruzi GP72 is 44% identical and 63% similar to
T. brucei FLA1 at the amino acid level (see Fig. 8A). As with
FLA1 in T. brucei, GP72 is localized to the junction between the
flagellum and the cell body and is required for flagellar attach-
ment in T. cruzi. However, GP72 is not required for T. cruzi
viability in cell culture. In contrast to T. brucei FLA1, both
alleles of T. cruzi GP72 can be deleted from the genome with no
obvious deleterious effects on growth in cell culture. The re-
resultant T. cruzi with detached flagella are immobile but con-
tinue to divide normally (16). We were therefore interested in
whether T. cruzi GP72 could compensate for the flagellar de-
thachment or cytokinesis defects in T. brucei expressing FLA1
dsRNA. To address this question, we expressed GP72 in the
procyclic p2T7TiA/FLA1 cell line.

GP72 and GFP were PCR-amplified and inserted into a modified
version of the pXS2:pac expression vector (the kind gift of
Dr. J. Bangs, University of Wisconsin, see Ref. 20) to produce
plasmids pSk1-GP72 and pSk1-GFP. Because we intended to
integrate these plasmids into the genomes of cells expressing
T7 RNA polymerase, we removed the T7 promoter, which could
potentially generate antisense RNA, from pXS2:pac (pSk1).
pXS2:pac and the derivatives described above integrate into the
TUB locus and drive the expression of the heterologous
sequences via the EP-PARP promoter. Linearized pSk1-GP72
and pSk1-GFP were individually introduced into procyclic
2T7T1A/FLA1, p2T7T1A/GFP, and parental 29-13 cells, and stable cell lines were selected in the presence of puromycin. Northern blots probed with the GP72 coding sequence verified that GP72 was expressed in these cells (Fig. 6A).

Unexpectedly, the expression of GP72 caused flagellar detachment in the absence of added tetracycline in all three cell lines that contained pSk1-GP72 (Fig. 6B and Table 1). This effect was specific to the expression of GP72; cell lines containing pSk1-GFP exhibited green fluorescence but not flagellar detachment. We also observed detached flagella in wild-type YTAT 1.1 procyclic T. brucei that were transiently transfected with pSk1-GP72 (not shown), confirming that the detached flagella were due to GP72 expression and that the effect was neither strain-specific nor due to the site of integration.

As described under “Experimental Procedures,” we have verified by DNA sequence determination that the GP72 sequence in this construct is the wild type. Thus, T. cruzi GP72 dominantly interferes with the T. brucei flagellum attachment in procyclic T. brucei trypanosomes.

Perhaps the most surprising aspect of this result is that we were able to obtain stable cell lines of procyclic T. brucei that had detached flagella. As shown in Fig. 4A, inhibition of FLA1 expression by RNAi in procyclic cells resulted in detached flagella, but these cells were unable to divide. In contrast to cells in which FLA1 was depleted by RNAi, the pSk1-GP72 cell lines were able to grow despite having completely detached flagella, albeit at a 2- to 3-fold reduced rate as compared with the equivalent cell lines containing pSk1-GFP (data not shown). The reduced growth rate provided a strong negative selection, however, and cells with wild-type morphology eventually grow out of these populations. This effect was most dramatic in parental 29-13 cells (Table 1). For unknown reasons, only about one-third of puromycin-resistant 29-13 cells transfected with linear pSk1-GP72 had detached flagella, and cells with attached flagella eventually outgrew those with detached flagella. In contrast, greater than 90% of the puromycin-resistant cells that were obtained from transfections of 2T7T1A/FLA1 and p2T7T1A/GFP with linear pSk1-GP72 had detached flagella, although this percentage also decreased over time.

Although GP72 interfered with FLA1 function, it was still possible that GP72 might be able to mediate flagellar attachment in the absence of FLA1. To determine whether GP72 could substitute for FLA1, the expression of FLA1 dsRNA was induced by adding tetracycline to the cultures. As shown in Table 1, the percentage of cells with detached flagella remained constant through 48 h after adding tetracycline. We have shown previously that RNA levels are reduced to nearly undetectable levels within 4 h after inducing dsRNA expression with tetracycline in the 2T7T1 system (22) and that detached flagella appear by 18 h after adding tetracycline. Thus, by 48 h, little FLA1 is likely to remain in the 2T7T1A/FLA1 cells. The fact that these cells did not develop attached flagella suggests that GP72 cannot substitute for FLA1 in procyclic T. brucei or that once detached, the flagellum cannot reattach.

Expression of FLA1 dsRNA in Bloodstream Trypanosomes Causes Flagellar Detachment—To determine whether FLA1 plays the same role in bloodstream T. brucei as in procyclic T. brucei, p2T7T1A/FLA1 and p2T7T1A/GFP were integrated into the genome of the single marker bloodstream T. brucei cell line that co-expresses T7 RNA polymerase and the tetracycline repressor (18). When grown in the presence of tetracycline, more than 90% of 2T7T1A/FLA1 bloodstream cells exhibited detached flagella (Fig. 7A). Bloodstream cells with detached flagella appeared 6–8 h after adding tetracycline, about 12 h earlier than in procyclic cells. Some leaky expression was evident as ~5% of the uninduced cells also had detached flagella. In contrast, clonal lines with an integrated copy of p2T7T2/AGFP appeared normal in the absence or presence of tetracycline (Fig. 7A), demonstrating that flagellar detachment depended on the expression of FLA1 dsRNA.

To verify that FLA1 expression was being inhibited, we analyzed FLA1 RNA in bloodstream T. brucei 2T7T2/AGFP and 2T7T2/AGFP cells on northern blots (Fig. 7B). As expected, the level of the ~3-kb FLA1 RNA was similar in 2T7T2/AGFP cells grown in the absence or presence of tetracycline and in 2T7T2/AGFP cells grown in the absence of tetracycline. When tetracycline was added to the 2T7T2/AGFP cultures, the level of FLA1 RNA was reduced. A low level of FLA1 RNA remained in these cultures, most likely due to reversion of 2T7T2/AGFP to the wild type. Reversion of bloodstream 2T7T2/AGFP cells to a tetracycline non-responsive phenotype occurred more rapidly.

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**Table 1**

| Parental cell line | Expression construct | % with detached flagella
|--------------------|----------------------|------------------------
| 29–13              | none                 | <1                     |
| 29–13              | GP72                 | 36                     |
| 2T7T1A/FLA1        | none                 | 98                     |
| 2T7T1A/FLA1        | GP72                 | 98                     |
| 2T7T1A/AGFP        | GP72                 | 98                     |

- Tetrazycline (1 μg ml⁻¹) was added to the culture media at time zero. The column marked “−tet” indicates the percentage of cells with detached flagella prior to adding tetracycline. A minimum of 200 cells was counted.
- The parental cell lines in the left-hand column were transfected with plasmid pSk1-GP72 (GP72) or pSk1-GFP (GFP), and stable cell lines were obtained. “None” indicates that no additional plasmids were inserted into the genome.
more extensively. The most obvious difference between the two proteins is the presence of a 44-aa amino acid proline-rich insertion in the middle of the FLA2 protein. Because FLA1 and FLA2 are nearly identical over their first 300 nucleotides and because this region was included in the FLA1 probe used in Fig. 7B, we predict that the higher molecular weight band on the northern blot corresponds to FLA2. Similarly, because this same FLA1 sequence was included in p2T7^Tg/FLA1, both FLA1 and FLA2 will be inhibited by the FLA1 dsRNA. Thus, the phenotypes generated by FLA1 RNAi in bloodstream T. brucei may be due to either FLA1 or FLA2 acting individually or in concert.

Expression of FLA1 dsRNA Inhibits Cell Division in Bloodstream Trypanosomes—We next measured the growth rates of bloodstream 2T7^Tg/GFP and 2T7^Tg/FLA1 cells in the presence and absence of tetracycline (Fig. 4B). As observed with procyclic cultures, addition of tetracycline did not affect the growth of bloodstream T. brucei containing the p2T7^Tg/GFP construct. However, the growth of bloodstream 2T7^Tg/FLA1 cells was severely reduced in the presence of tetracycline. As compared with procyclic 2T7^Tg/FLA1 cultures, bloodstream 2T7^Tg/FLA1 cells were affected more rapidly by the addition of tetracycline. Bloodstream 2T7^Tg/FLA1 cultures failed to double even once after adding tetracycline, whereas procyclic lines double approximately twice before reaching a plateau. Bloodstream 2T7^Tg/FLA1 also developed multiple nuclei and kinetoplasts more rapidly than their procyclic counterparts (Fig. 9). Multinucleated cells were not detected in untreated 2T7^Tg/FLA1 cultures or in 2T7^Tg/GFP cells grown in the presence or absence of tetracycline, indicating that this effect was due to the expression of FLA1 dsRNA. Thus, this experiment demonstrates that FLA1 and/or FLA2 are required for cell division but not mitosis or kinetoplast replication in bloodstream T. brucei. Further experiments are needed to dissect the individual roles of FLA1 and FLA2 in bloodstream trypanosomes.

DISCUSSION

We show here that inhibiting FLA1 expression in procyclic T. brucei by RNAi causes flagellar detachment and blocks cell division. Despite this block in cytokinesis, mitosis continues, and the cells develop multiple nuclei, demonstrating that in T. brucei, cytokinesis and mitosis are not linked processes. Surprisingly, the expression of T. cruzi GP72 in procyclic T. brucei also caused flagellar detachment, but in this case, the cells with detached flagella continued to divide. These data suggest that flagellar attachment is not absolutely necessary for cytokinesis and that FLA1 has two separable roles in procyclic cells, one in flagellar attachment and one in cytokinesis. It is not clear whether these roles represent discrete biochemical properties or distinct concentration dependences (i.e. high levels of FLA1 may be needed for flagellar attachment, but lower levels may be required for cytokinesis).

The ability of procyclic T. brucei expressing T. cruzi GP72 to undergo cytokinesis is unexpected based on the recent discovery of the flagellar complex (24). This novel trypanosome structure links the tip of the newly forming flagellum to the old flagellum and has been hypothesized to transmit information needed to replicate the helical pattern of African trypanosomes and to maintain cell polarity (24). However, procyclic T. brucei expressing GP72 have completely detached old and new flagella yet are still able to divide. Even if the flagellar complex is able to form in these cells, the complex cannot transmit any positional information to the cell body if it is not attached. This observation suggests that flagellar attachment per se and the flagellar complex are not essential for cytokinesis to occur. However, GP72-expressing cells grew more slowly than controls and were eventually out-competed by cells with wild-type
morphology, suggesting that flagellar attachment or the flagellar complex are needed for efficient cell division.

The key question that emerges from these experiments is how FLA1 might be involved in cytokinesis. Characterization of a similar T. brucei FLA1-RNAi cell line revealed that the FAZ is improperly formed in cells with a detached flagellum (24). Given the invariant location of the FAZ, this structure has been proposed to provide positional cues for cleavage during cell division (9). Taken together, these experiments suggest that the cytokinesis defect caused by the loss of FLA1 is due to the improperly formed FAZ. FLA1 appears to be at the right place at the right time to influence the creation of the new FAZ. FLA1 has been localized by immunofluorescence to the flagellar pocket and to the region of flagellar attachment to the cell body and thus appears to be in close physical proximity to the FAZ filament (16). In addition, synthesis of the new flagellum appears to precede the construction of the new FAZ (8). However, it is unclear how FLA1 could affect the formation of the FAZ since the vast majority of the FLA1 protein is predicted to be extracellular. FLA1 has an amino-terminal signal sequence predicted to direct it to the secretory system and a single transmembrane domain near the carboxyl terminus. Only 16 amino acids are predicted to be found in the cytoplasm, making it unlikely that FLA1 plays an extensive structural role in the FAZ filament. A potential explanation is that FLA1 may be involved in specifying the location of the FAZ. As described above, the recently identified flagellar complex is proposed to transmit positional cues that determine the site and direction

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**Fig. 8. Sequence alignment of T. brucei FLA2 and FLA1 and T. cruzi GP72.** Sequences were aligned using ClustalW. Amino acids conserved in two or more proteins are shaded in black. Dashes indicate gaps. The asterisks indicate conserved cysteines. The predicted transmembrane domains are underlined.

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**Fig. 9. Expression FLA1 dsRNA in bloodstream T. brucei causes the formation of multinucleated cells.** Bloodstream 2T7TiA/FLA1 (A and B) and 2T7TiA/GFP (C and D) were grown in the presence of tetracycline (0.75 μg ml⁻¹, 30 h), fixed, and stained with DAPI to visualize nuclei and kinetoplasts. DIC and fluorescent images were obtained from the same field using a Zeiss Axiosplan 2 microscope equipped with a x100 oil immersion lens and an RT spot camera. The scale bar indicates 10 μm.
of the cleavage furrow. The ability of the flagellar complex to transmit this information appears to depend on FLA1 since the FAZ is malformed in cells that do not express FLA1 (24). FLA1 could be directly involved in specifying the FAZ, possibly through interactions with its carboxyl-terminal cytoplasmic tail. In this model, the flagellar complex would specify the localization or deposition of FLA1, which in turn would define the position of the FAZ. Alternatively, FLA1 could be indirectly involved in FAZ formation by maintaining flagellar attachment and allowing the flagellar complex to more efficiently direct construction of the FAZ filament.

Inhibition of Flagellum Attachment by T. cruzi GP72—The most surprising finding described here is that the expression of T. cruzi GP72 in procyclic T. brucei causes flagella to detach from the cell body. T. cruzi GP72 and T. brucei FLA1 have significant sequence similarity throughout their entire length, and each is required for flagellar attachment in its respective organism. However, in contrast to FLA1, GP72 is required only for flagellar attachment in T. cruzi epimastigotes but is dispensable for cytokinesis (13). Consistent with this observation, GP72 expression in procyclic T. brucei caused flagellar detachment but did not prevent cell division.

The mechanism by which T. cruzi GP72 causes flagellar detachment in procyclic T. brucei remains to be determined. Given the homology between GP72 and FLA1, one likely explanation is that GP72 dominantly interferes with FLA1 function. If so, the interference appears to specifically affect the role of FLA1 in flagellar attachment but not its function in cytokinesis. GP72 might mediate its dominant interference by oligomerizing with FLA1 and preventing proper FLA1 localization or function. Alternatively, GP72 may bind to FLA1-interacting proteins and prevent their binding to FLA1.

Identification of FLA2—We have identified a novel FLA1-related protein (FLA2) expressed in bloodstream T. brucei. Based on sequence information currently available in the GenBank™ HTGS data base, FLA2 appears to have arisen as a gene duplication event on chromosome 8 that included FLA1 and at least 2500 bp upstream. In addition to FLA1 and FLA2, two other open reading frames were also duplicated, one of which is duplicated identically. Neither of these open reading frames have significant homology to any known proteins, and thus, their functions are unclear. Similarly, the function of FLA2 is also unclear. The phenotypes of bloodstream and procyclic T. brucei expressing FLA1 dsRNA are identical, at least for the parameters investigated in this report. Since procyclic T. brucei express FLA1 but do not appear to express FLA2, FLA1 is apparently sufficient to account for the defects in flagellar attachment and cytokinesis observed in both procyclic and bloodstream 2TTΔA/FLA1 cell lines. However, because the FLA1 sequence in p2TTΔA/FLA1 included 300 bp of sequence that was identical to FLA2, both FLA1 and FLA2 were inhibited in bloodstream 2TTΔA/FLA1 cells. Thus, we cannot rule out the possibility that FLA2 also plays a role, if indeed a FLA2 protein is produced. Perhaps FLA1 and the putative FLA2 are functionally redundant or there are slight differences in the architecture or mode of duplication of bloodstream T. brucei that necessitate both proteins. Further experiments will be needed to distinguish among these possibilities and to determine whether the putative FLA2 can functionally substitute for FLA1 in procyclic T. brucei cells or in T. cruzi lacking GP72.

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REFERENCES

1. Bastin, P., Matthews, K. R., and Gull, K. (1996) Parasitol. Today 12, 302–307
2. Mags, A. J., Sherwin, T., Francis, S., Gull, K., and LeBowitz, J. H. (1999) J. Cell Sci. 112, 2753–2763
3. Bastin, P., Sherwin, T., and Gull, K. (1998) Nature 391, 548
4. Kohl, L., and Gull, K. (1998) Mol. Biochem. Parasitol. 93, 1–9
5. Sherwin, T., and Gull, K. (1989) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 325, 573–588
6. Gull, K. (1999) Annu. Rev. Microbiol. 53, 629–655
7. Woodward, R., and Gull, K. (1996) J. Cell Sci. 95, 45–57
8. Kohl, L., Sherwin, T., and Gull, K. (1999) J. Eukaryot. Microbiol. 46, 105–109
9. Robinson, D. R., Sherwin, T., Plouhidou, A., Byard, E. H., and Gull, K. (1995) J. Cell Biol. 126, 1163–1172
10. Ferguson, M. A. J., Allen, A. K., and Snary, D. (1983) Biochem. J. 213, 313–319
11. Cooper, R., Inverso, J. A., Espinosa, M., Nogueira, N., and Cross, G. A. (1991) Mol. Biochem. Parasitol. 49, 45–59
12. Haynes, P. A., Russell, D. G., and Cross, G. A. (1996) J. Cell Sci. 109, 2979–2986
13. Cooper, R., de Jesus, A. R., and Cross, G. A. (1993) J. Cell Biol. 122, 149–156
14. Ribeiro de Jesus, A., Cooper, R., Espinosa, M., Gomes, J. E., Garcia, E. S., Paul, S., and Cross, G. A. (1993) J. Cell Sci. 106, 1023–1033
15. el-Sayed, N. M., Alarcon, C. M., Beck, J. C., Sheffield, V. C., and Donelson, J. E. (1995) Mol. Biochem. Parasitol. 73, 75–90
16. Nozaki, T., Haynes, P. A., and Cross, G. A. (1996) Mol. Biochem. Parasitol. 82, 245–255
17. LaCount, D. J., Bruse, S., Hill, K. L., and Donelson, J. E. (2000) Mol. Biochem. Parasitol. 111, 67–76
18. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
19. Hill, K. L., Hutchings, N. R., Russell, D. G., and Donelson, J. E. (1999) J. Cell Sci. 112, 3081–3091
20. Bangs, J. D., Brouch, E. M., Ransom, D. M., and Roggy, J. L. (1996) J. Biol. Chem. 271, 14877–14883
21. Hirumi, H., Hirumi, K., Doyle, J. J., and Cross, G. A. (1980) J. Mol. Biochem. Parasitol. 309, 1163–1172
22. LaCount, D. J., and Donelson, J. E. (2001) Protist 152, 103–111
23. Wang, Z., Morris, J. C., Drew, M. E., and Enghuld, P. T. (2000) J. Biol. Chem. 275, 40174–40179
24. Noguera-Leite, F. F., Sherwin, T., and Gull, K. (2001) Science 294, 610–612
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