Trypanosoma brucei is a protozoan parasite that causes African sleeping sickness. T. brucei multiplies extracellularly in the bloodstream, relying on antigenic variation of a dense variant surface glycoprotein (VSG) coat to escape antibody-mediated lysis. We investigated the role of VSG in proliferation and pathogenicity by using inducible RNA interference to ablate VSG transcript down to 1–2% normal levels. Inhibiting VSG synthesis in vitro triggers a rapid and specific cell cycle checkpoint blocking cell division. Parasites arrest at a discrete precytokinesis stage with two full-length flagella and opposing flagellar pockets, without undergoing additional rounds of S phase and mitosis. A subset (1%–10%) of the stalled cells have internal flagella, indicating that the progenitors of these cells were already committed to cytokinesis when VSG restriction was sensed. Although there was no obvious VSG depletion in vitro after 24-h induction of VSG RNA interference, there was rapid clearance of these cells in vivo. We propose that a stringent block in VSG synthesis produces stalled trypanosomes with a minimally compromised VSG coat, which can be targeted by the immune system. Our data indicate that VSG protein or transcript is monitored during cell cycle progression in bloodstream-form T. brucei and describes precise precytokinesis cell cycle arrest. This checkpoint before cell division provides a link between the protective VSG coat and cell cycle progression and could function as a novel parasite safety mechanism, preventing extensive dilution of the protective VSG coat in the absence of VSG synthesis.

**African trypanosomes** are unicellular parasites transmitted by tsetse flies in sub-Saharan Africa, which are responsible for debilitating disease in humans and livestock. African sleeping sickness is resurgent, with an estimated 300,000–500,000 cases a year, and it is invariably fatal if untreated (1). T. brucei multiplies extracellularly in the bloodstream, exposing it to immune attack. Each bloodstream-form cell is coated with a dense layer of VSG molecules of a given antigenic type, making the VSG coat a potential drug target for trypanosomiasis.

**Materials and Methods** Trypanosome Strains and Culture. VSG RNAi was performed in T. brucei 427 90-13 expressing VSG221 (6). These trypanosomes were transfected with the MC177/VSG221 RNAi construct containing an 803-bp VSG221 target fragment (GenBank accession no. X56762, positions 122–925) inserted between the opposing T7 promoters of construct p27T7-177 (7). To ensure that the transformants did not switch away from the VSG221 expression site (ES), the 221GPI construct containing GFP and puromycin (8) was inserted immediately behind the 221 ES promoter, resulting in the T. brucei 221VG1.1 and 221VG2.1 cell lines. Alternatively, a blasticidin resistance gene was inserted behind the VSG221 ES promoter, resulting in T. brucei 221VB1.1 or 221VB2.1. T. brucei was cultured in vitro in HMI-9 medium (9) or in vivo in CBA/CA mice (Harlan UK, Bicester, U.K.). All results presented were comparable between the T. brucei 221VG1.1 and 221VG2.1 and the T. brucei VB1.1 and VB2.1 cell lines.

**T. brucei Microscopy.** Cells for cell cycle analysis by microscopy were fixed at an end concentration of 2% paraformaldehyde, allowed to settle on glass slides, washed, and mounted in Vectashield containing 40 ng/ml DAPI (Vector Laboratories). For cell cycle analysis, an average of 1,200 cells was counted per time point. Cells for immunofluorescence were fixed comparably, but then reacted with an anti-VSG221 rabbit polyclonal antibody (gift of P. Borst’s laboratory) and an anti-rabbit secondary antibody coupled to Alexa 488 (Molecular Probes). Microscopic analysis was performed with a Zeiss Axioplan 2 microscope. Images were obtained with a Roper Scientific (Trenton, NJ) Cool Snap HQ camera and analyzed with METAMORPH 6.20 imaging software (Universal Imaging, Downingtown, PA).

Endocytosis was followed by incubating cells in 10 μg/ml−1
of FITC-tomato lectin. Endocytosis ceases at 4°C. To probe for its activation, cells were transferred to 37°C for 4 min to allow internalization of the FITC-tomato lectin (9). Endocytosis experiments were performed with the T. brucei 221VB1.1 or 221VB2.1 transformants, which stalled in an equivalent fashion to the T. brucei 221VG1.1 and 221VG2.1 transformants after induction of VSG221 RNAi. Cells were prepared for scanning electron microscopy or transmission electron microscopy essentially as described (10, 11) except that the scanning electron microscopic cells were fixed in the culture medium.

**T. brucei Analysis.** RNA analysis was performed according to ref. 8. The VSG221 probe was the fragment used for VSG RNAi. Lysates for protein analysis were made by washing cells and then resuspending them at 10^9 cells per ml in cold lysis buffer [50 mM Hepes, pH 7.5/1.5 mM MgCl_2/1 mM EDTA/10% glycerol/1% Triton X-100] one mini protease inhibitor mixture tablet (Roche Diagnostics) per 10-ml mix]. After lysis and microcentrifuge centrifugation, the supernatants were analyzed further. Western blotting was performed by using standard protocols and antibodies against VSG221 (gift of P. Borst) or BiP (gift of Jay Bangs, University of Wisconsin, Madison). Flow cytometry was performed according to ref. 8. Cell volume measurements were carried out by using the Coulter size exclusion method on a CASY cell counter model TT (Schäffer, Reutlingen, Germany) according to the manufacturer’s instructions.

**Results and Discussion**

**RNAi-Mediated Ablation of VSG Transcript Results in Rapid Growth Arrest in Vitro.** To address the functional role of VSG, we specifically inhibited VSG synthesis by performing RNAi against the VSG expressed by our variant of the T. brucei 427 strain (VSG221) by using a tetracycline-inducible construct containing a fragment of VSG221 (6, 12). Transformants were obtained only by using an experimental strategy optimized for extremely low transcriptional read-through (7). Surprisingly, induction of VSG221 RNAi in vitro led to a very rapid inhibition of cell growth within one cell cycle (8 h) (Fig. 1A). This finding indicates that VSG has an essential function even in vitro in the absence of an immune system. VSG mRNA is one of the most abundant transcripts in T. brucei, yet after only 4-h induction of VSG RNAi it was reduced to 1–2% of normal levels (Fig. 1B). After tetracycline induction we did not detect dsRNA corresponding to VSG221, presumably because of very efficient processing by the RNAi machinery. After initial growth arrest, cells eventually grew out after ~84 h. Analysis of 50 RNAi “escape” clones isolated in the absence of drug selection pressure on the active ES showed that 49 of 50 had switched to different VSG coats not recognized by the VSG221 RNAi rather than had inactivated the RNAi machinery (results not shown). As expected, if drug selection pressure on the active ES was maintained during induction of VSG RNAi, VSG switch variants were generated that had exclusively switched via DNA rearrangements (results not shown). These experiments argue for specificity of the
VSG221 RNAi, as cells that express a new VSG escape the growth arrest. RNAi against a GFP gene inserted into the active VSG221 ES did not lead to growth defects (result not shown). Similarly, RNAi-mediated ablation of the different ES/4G transcription units within the VSG221 ES did not lead to a comparable growth arrest (result not shown), again arguing that the precytokinesis arrest that we observe is specific to VSG and that we are not merely disrupting a polycistronic transcription unit.

Despite the radical reduction in VSG transcript after VSG RNAi, cells remained VSG221-positive with no observable decrease of VSG on the cell surface as monitored by immunofluorescence, cell volume microscopy (Fig. 1C) or flow cytometric analysis (results not shown) with anti-VSG221 antibodies. In addition, there was no significant reduction in total VSG levels within 24 h of arrest as assayed by Western blot using dilutions of protein lysates and BiP as a loading control (Fig. 1) (13). VSG has an extremely long half-life (33 ± 9 h) (14) and shows rapid lateral diffusion within the coat (15), presumably explaining why cells with reduced VSG synthesis remain viable for extensive periods in vitro. In some respects this longevity mimics stumpy-form trypanosomes, which are a nonreplicating form induced at high cell densities in a normal bloodstream infection and are able to survive for days despite having halted VSG synthesis (16, 17).

**Cells Stalled After Inhibition of VSG Synthesis Arrest at a Precise Precytokinesis Stage.** Given the very fast and effective growth arrest after the induction of VSG RNAi, we asked whether individual cells exhibited specific cell cycle phenotypes. DNA staining with DAPI allows analysis of *T. brucei* cell cycle stages. *T. brucei* has one nucleus (N) and kinetoplast (K) (mitochondrial genome), and post-S phase, the kinetoplast divides before the nucleus (18). After induction of VSG221 RNAi in vitro, cells rapidly accumulate at a 2K2N precytokinesis stage (Fig. 2A). This finding indicates that inhibiting VSG synthesis triggers a specific cell cycle checkpoint. Cell cycle arrest is highly effective, with no significant accumulation of multinucleated cells characteristic of successive rounds of S phase and mitosis. Flow cytometric analysis of propidium iodide-stained cells stalled after VSG RNAi indicated that these cells had not initiated additional rounds of S phase after induction of the precytokinesis cell cycle arrest (Fig. 2B). These results present an example of a precise precytokinesis arrest in trypanosomes. Such cell cycle arrest could operate to allow time for correction for fluctuations in VSG synthesis.

The trypanosome builds a new flagellum and flagellar pocket in its cell cycle, and flagella are instrumental in kinetoplast segregation. We used scanning electron microscopy to compare typical precytokinesis trypanosome stages in cultures that were uninhibited or induced for VSG RNAi for 24 h (Fig. 3). Cells arrested after the induction of VSG RNAi had two full-length flagella, each with its own flagellar pocket at the base of the flagellum, but lacked a deep cleavage furrow (Fig. 3). In contrast to normal precytokinesis stages, cells stalled after VSG RNAi were characteristically shorter and broader (Fig. 3 and Fig. 7A, which is published as supporting information on the PNAS website). The average volume of uninhibited *T. brucei* VSG221 cells is ~24 μm³ as measured by a Coulter counter, and this volume doubles in cultures induced for VSG RNAi for 12 h (Fig. 8, which is published as supporting information on the PNAS website). As precytokinesis trypanosomes are expected to have double the volume of cells present in the modal peak of an uninhibited culture, cells stalled after VSG RNAi are equivalent in volume to normal precytokinesis stages. The shorter and broader morphology of cells stalled after VSG RNAi could be a consequence of the trypanosomes attempting to minimize the surface area-to-volume ratio in the face of restricted VSG.

A typical feature of trypanosome-arrested precytokinesis by VSG RNAi was the reduced distance between the two kinetoplasts and nuclei (Fig. 7B and C). This distance is characteristic of the bloodstream-form *T. brucei* “phase 5” stage of the cell cycle immediately before cell division (19). Trypanosomes stalled precytokinesis by VSG RNAi typically had parallel flagella and opposed flagellar pockets (Fig. 3), again suggesting arrest at a precise precytokinesis point. This configuration could also be found in the uninhibited population, but very infrequently, indicating that VSG RNAi has resulted in a cell cycle block, resulting in the accumulation of a normally transient stage in the cell cycle.

**Endocytosis Is Unimpaired in Arrested Cells.** Bloodstream-form *T. brucei* has one of the highest rates of endocytosis measured, with the entire pool of surface VSG recycled every 12 min (20, 21). This very high rate of endocytosis, which protects the trypanosome by removing anti-VSG antibody complexes, is restricted to
the flagellar pocket (21). We therefore asked whether both flagellar pockets were functional for endocytosis in these trypanosomes arrested by VSG RNAi. FITC-tomato lectin binds flagellar pocket glycoproteins and can be used as a probe for the endocytic pathway (9). Endocytosis stops at 4°C (22), and incubation of stalled T. brucei cells with FITC-tomato lectin at 4°C labeled the two flagellar pockets, with a slightly smaller signal in the new flagellar pocket as in the controls (Fig. 4). Endocytosis into the cell reactivates upon subsequent incubation at 37°C (22), resulting in tracks of lectin-labeled vesicles extending from the flagellar pockets, again as in controls. Therefore, both flagellar pockets can be functionally active in these arrested cells.

**VSG RNAi Results in Cells with Internal Flagella.** The VSG coat was originally defined by electron microscopy as an electron-dense layer coating bloodstream-form T. brucei (23). We confirmed the apparent integrity of the VSG coat on the many stalled precytokinesis cells in the VSG RNAi-induced population by using this approach. After 24 h of VSG RNAi induction, the VSG coat on cross sections of stalled cells looked comparable to controls (Fig. 5A). We further investigated the subcellular architecture of the VSG RNAi cells by using electron microscopy. The majority of noninduced T. brucei had one external flagellum (Fig. 5B). After induction of VSG RNAi, cells accumulated with two external flagella (indicated by arrows in Fig. 5C). Further, we saw no disruption of the subpellicular microtubule corset after 24 h of VSG RNAi, and both flagella were associated with the normal cytoplasmic structures of the flagellum attachment zone (Fig. 5C). We did not detect systematic changes in endomembrane organization. In a subset (<10%) of the VSG RNAi-induced population, trypanosomes were seen with one external and one internal flagellar axoneme and associated paraxflagellar rod (Fig. 5D). We propose that VSG RNAi is so effective that the majority of the cells implement the precytokinesis checkpoint within one cell cycle (Fig. 9A), which is published as supporting information on the PNAS web site). However, cells committed to cytokinesis upon induction are likely to divide before the RNAi is fully effective (Fig. 9B). After cell division, these trypanosomes will be experiencing a major reduction in VSG coat synthesis some 4 h later when they initiate new flagellum formation. We suggest that their new basal body fails to make a productive connection with the cell surface, or there is insufficient VSG-coated surface membrane, and hence the new flagellum is extended internally in the cytoplasm (Fig. 9B). This phenomenon reveals that although surface membrane/coat and subpellicular microtubule cytoskeleton construction is normally coordinated, there is no dependency relationship between the two. Cytoskeletal assembly can sometimes continue extensively in the face of this VSG restriction, such that occasionally at later stages cells exhibit cytoplasmically located microtubule bundles along with multiple internal flagella (Fig. 5E).

**VSG Is Essential in Vivo.** Finally, we addressed the role of the VSG coat in pathogenicity by establishing infections in mice (Fig. 6). Growth of the parental T. brucei 90-13 strain lacking the VSG221 RNAi construct was not affected by addition of the tetracycline analogue doxycycline to the drinking water (Fig. 6A) (24). We next infected six mice with T. brucei 221VG1.1 and induced VSG RNAi by using doxycycline in half of the mice 64 h after inoculation. Although there was no significant reduction of the VSG221 coat detectable in vitro after VSG RNAi, induction of VSG RNAi in vivo resulted in rapid clearance of the infection within 12 h. This finding argues that even minor, and experimentally not easily detectable, damaging of the VSG coat is fatal for the parasite, and that a fully uncompromised VSG coat is essential for successful infection. We postulate that in the absence of VSG synthesis, even in the presence of a cell cycle arrest, chinks form in the protective layer that can be exploited by an immunocompetent mouse. These results were comparable.
with different T. brucei VSG221 RNAi transformants and mouse strains (results not shown).

The rapid clearance of parasites must be caused by recognition by the immune system or a mouse lytic factor, rather than a consequence of the cell growth arrest. The stumpy forms of T. brucei are naturally occurring nondividing forms that are arrested in their cell cycle during an infection. However, stumpy-form trypanosomes possess an intact VSG coat, and although nonproliferative, have a longevity between 48 and 72 h (25). Therefore, cell cycle arrest on its own would not lead to the dramatic clearance kinetics that we observe. Stumpy-form T. brucei are fundamentally different from the slender bloodstream form by the immune system or a mouse lytic factor, rather than a component of the mouse immune response instead of antibody-mediated lysis is involved in clearance. One possibility is that there is increased complement deposition on the surface of trypanosomes stalled after the induction of VSG RNAi, which could result in increased clearance of these trypanosomes by macrophages. This scenario, however, still remains to be tested. At the moment we cannot exclude that the trypanosomes stalled by VSG RNAi are cleared by a mouse lytic factor that is not part of the complement system.

Cell Cycle Arrest in T. brucei. Why are the trypanosomes that are stalled after VSG RNAi cleared in vivo despite the presence of a cell cycle checkpoint preventing significant depletion of the VSG coat? Inhibiting VSG synthesis triggers a cell cycle checkpoint, which could have evolved to absorb less drastic fluctuations in VSG synthesis than are occurring in our VSG RNAi experiments. In our experiments VSG RNAi resulted in an ablation of VSG transcript to <1–2% normal levels, presumably more disruption than can be absorbed without small but lethal chinks appearing in the coat that can be exploited by the immune system of the mouse.

Tsetse-form trypanosomes lacking the major surface molecule procyclin are viable and proliferate in vitro (27). In contrast, we show here that bloodstream-form T. brucei is acutely sensitive of the amount of its major surface molecule VSG, or of the VSG mRNA itself, as ablation of VSG transcript results in a rapid and specific precytokinesis block. Earlier studies have shown that disruption of the mitotic cyclin gene CYC6 in bloodstream-form T. brucei resulted in a cell cycle arrest before cell division, but this arrest was not precise, as cells reinitiated S phase and accumulated multiple kinetoplast DNA (28). Similarly, disruption of GPI8 encoding the catalytic subunit of a complex that adds glycosylphosphatidylinositol (GPI) anchors onto nascent polypeptides resulted in growth-arrested cells that had undergone repeated rounds of S phase and mitosis, resulting in the accumulation of “monster” cells with multiple nuclei, kinetoplasts, and flagella (29). This GPI8 disruption would prevent all GPI-anchored proteins (including VSG) from reaching the cell
checkpoint is triggered in the absence of VSG production, which is being "sensed," we find it more likely that this precytokinesis checkpoint has many hallmarks of the characteristic of a natural infection. In both cases, progression through the cell cycle could be linked to monitoring of VSG synthesis.

Although we have not excluded that it is the transcript that is being "sensed," we find it more likely that this precytokinesis checkpoint is triggered in the absence of VSG production, which would otherwise eventually lead to a disastrous dilution of the protective VSG coat. This process would provide a safety mechanism protecting the integrity of the VSG coat, which we show is vital for survival and pathogenicity in the mammalian host.

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**A**

Mean cell body length

![Bar chart showing mean cell body length at 0, 8h, and 24h.](image)

**B**

Mean interkinetoplast distance

![Bar chart showing mean interkinetoplast distance at 0, 8h, and 24h.](image)

**C**

Mean internuclear distance

![Bar chart showing mean internuclear distance at 0, 8h, and 24h.](image)

**Fig. 7**
<table>
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<tr>
<th>Time induction (h)</th>
<th>Pseudo-diameter (μm)</th>
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<th>Volume increase (%)</th>
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Cells are precytokinesis when VSG knockdown is sensed

- no cytokinesis
- 2K2N block
- 2 external flagella
- 1 old and 1 new

Cells are committed to cytokinesis

- cytokinesis occurs
- 1K1N 1K1N

- 2K2N block at the end of the next cell cycle.
  - 1 external old flagellum
  - 1 internal new flagellum

**Fig. 9**