

# A differential role for actin during the life cycle of *Trypanosoma brucei*

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**Actin is expressed at similar levels but in different locations in bloodstream and procyclic forms of *Trypanosoma brucei*. In bloodstream forms actin colocalizes with the highly polarized endocytic pathway, whereas in procyclic forms it is distributed throughout the cell. RNA interference demonstrated that in bloodstream forms, actin is an essential protein. Depletion of actin resulted in a rapid arrest of cell division, termination of vesicular traffic from the flagellar pocket membrane leading to gross enlargement of the pocket, loss of endocytic activity and eventually cell death. These results indicate that actin is required for the formation of coated vesicles from the flagellar pocket membrane, which is the first step in the endocytic pathway. Although loss of actin in procyclic cells did not affect growth, the *trans* region of the Golgi became distorted and enlarged and appeared to give rise to a heterogeneous population of vesicles. However, the flagellar pocket was not affected. These findings suggest that trypanosomes have different functional requirements for actin during the bloodstream and procyclic phases of the life cycle.**

*The EMBO Journal* (2004) 23, 780–789. doi:10.1038/sj.emboj.7600094; Published online 12 February 2004  
**Subject Categories:** cell & tissue architecture; microbiology & pathogens

**Keywords:** actin; endocytosis; *Trypanosoma brucei*

## Introduction

African trypanosomes, for example, *Trypanosoma brucei*, are extracellular protozoan parasites that cause sleeping sickness in humans and Nagana in cattle. The life cycle of these unicellular eukaryotes involves stage-specific forms in the mammalian host and the tsetse fly vector as well as different cell cycle forms adapted for proliferative and quiescent phases of growth (Vickerman, 1985). Changes in morphology are perhaps the most visible characteristics of these different

forms. As suggested by their descriptive titles, the long slender and short stumpy forms of the mammalian bloodstream stage, which represent proliferative and growth-arrested cells respectively, are easily distinguishable by microscopy (Balber, 1972; Black *et al.*, 1985; Reuner *et al.*, 1997). Similarly, the actively dividing procyclic form in the tsetse fly midgut is morphologically distinct from the quiescent metacyclic form of the salivary glands. These changes in cell shape must reflect changes in the cytoskeleton and ultrastructure of the trypanosome (Matthews and Gull, 1994; Matthews *et al.*, 1995). Recently, we described a protein kinase, TBPK50, that is expressed only in dividing trypanosomes and functionally complements the yeast Orb6 protein kinase (Verde *et al.*, 1998; García-Salcedo *et al.*, 2002). This kinase is linked to the organization of the yeast actin cytoskeleton, and repression of Orb6 resulted in the disorganization of this cytoskeleton and changes in cellular morphology. These considerations focused our attention on the characterization of actin in *T. brucei*.

Although actin is a well-characterized protein in higher eukaryotes, this is not the case for trypanosomes (Kohl and Gull, 1998). The gene in *T. brucei* encodes a protein with up to 95 and 70% identity, respectively, with other protozoan and metazoan actins (Ben Amar *et al.*, 1988). The only reported study of trypanosome actin was the use of antibodies raised against rabbit actin to detect a 43-kDa protein in *T. cruzi* and *Leishmania mexicana* (Mortara, 1989). However, other workers were unable to reproduce these results using heterologous antibodies (Shi *et al.*, 2000). The latter study also employed RNA interference (RNAi) to ablate the actin mRNA in procyclic forms of *T. brucei*. Surprisingly, given that actin is generally considered to be an essential protein, a sustained 10- to 20-fold downregulation of the actin mRNA did not produce any obvious effects on the growth of procyclic cells, which continued to divide at a normal rate in culture.

Recent studies indicate that actin plays an important role in endocytosis and intracellular transport, but the precise function of actin in these processes is unclear (Jeng and Welch, 2001; McPherson, 2002; Qualmann and Kessels, 2002; Stamnes, 2002). A striking feature of endocytosis and exocytosis in bloodstream forms of African trypanosomes is that this traffic is polarized and restricted to a small invagination of the plasma membrane at the base of the flagellum called the flagellar pocket (Webster, 1989; Balber, 1990; Overath *et al.*, 1997). This polarization probably occurs because the flagellar pocket lacks the tight array of microtubules associated with the rest of the plasma membrane, which would be inimical to the fusion and fusion events essential for vesicle traffic. Endocytic activity also appears to vary during the life cycle of *T. brucei*. Bloodstream forms are dependent on receptor-mediated uptake of host-derived factors for growth and have high rates of endocytosis, whereas endocytic activity in procyclic forms appears to be absent or significantly reduced (Overath *et al.*, 1997; Borst and Fairlamb, 1998;

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Received: 22 August 2003; accepted: 5 January 2004; Published online: 12 February 2004

Morgan *et al*, 2002). Here we show that in bloodstream forms of *T. brucei* actin colocalizes with the polarized endocytic pathway, while in procyclic forms actin is not polarized but is distributed throughout the cell. Repression of actin expression by RNAi in bloodstream forms resulted in a cessation in growth, loss of the endocytic activity and termination of vesicular traffic from the flagellar pocket membrane. In contrast, procyclic forms continued to grow and divide even when actin was no longer detectable. However, the *trans* region of the Golgi became distorted and enlarged in these cells. These findings suggest that trypanosomes have different functional requirements for actin during the bloodstream and procyclic phases of the life cycle.

## Results

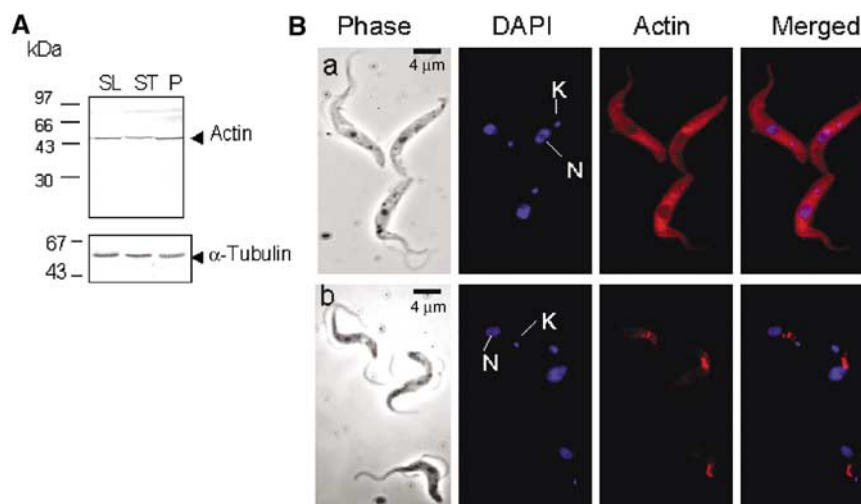
### Characterization of actin in bloodstream and procyclic forms of *T. brucei*

Different life-cycle forms of *T. brucei* (procyclic, bloodstream long slender and short stumpy forms) were subjected to Western blot analysis using polyclonal rabbit antibodies against a purified GST-actin fusion protein. These antibodies revealed the presence of a single protein in all stages that migrated with an apparent molecular weight of 44 kDa, in agreement with the size predicted from the gene sequence (Figure 1A). The level of expression was similar in all forms. The location of actin was investigated by indirect immunofluorescent antibody staining of fixed cells (Figure 1B). In procyclic forms, actin appeared to be uniformly distributed throughout the cell. In contrast, in bloodstream long slender forms, actin was primarily located in the posterior end of the cell and was clearly concentrated between the nucleus and kinetoplast. A similar polarized distribution was observed in bloodstream stumpy forms (data not shown). This region of the cell is the site of the highly active endocytic pathway in bloodstream trypanosomes. Therefore, colocalization studies were performed with tomato lectin, an established marker for the endocytic pathway in *T. brucei*, which binds specifically

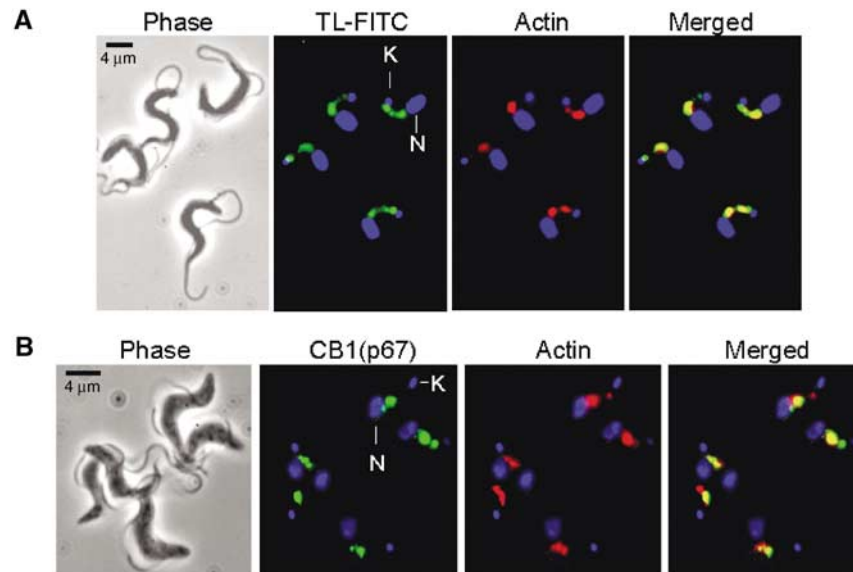
to glycoproteins containing poly-*N*-acetylglucosamine (PNAL) (Nolan *et al*, 1999). Actin colocalized with tomato lectin (Figure 2A) indicating that this protein is distributed throughout the endocytic pathway. Interestingly, Western blot analysis revealed the presence of a small amount of actin in the tomato lectin binding fraction isolated from bloodstream trypanosomes under nondenaturing conditions (data not shown). This finding suggested that at least some of the actin might be associated with protein complexes that contain PNAL. Actin also colocalized with the lysosomal membrane glycoprotein p67 as shown by the use of a monoclonal antibody against the CB1 epitope of this protein (Brickman and Balber, 1993; Kelley *et al*, 1999) (Figure 2B). These results demonstrated that the subcellular location of actin varies during the life cycle of trypanosomes.

### Actin is an essential protein in bloodstream forms

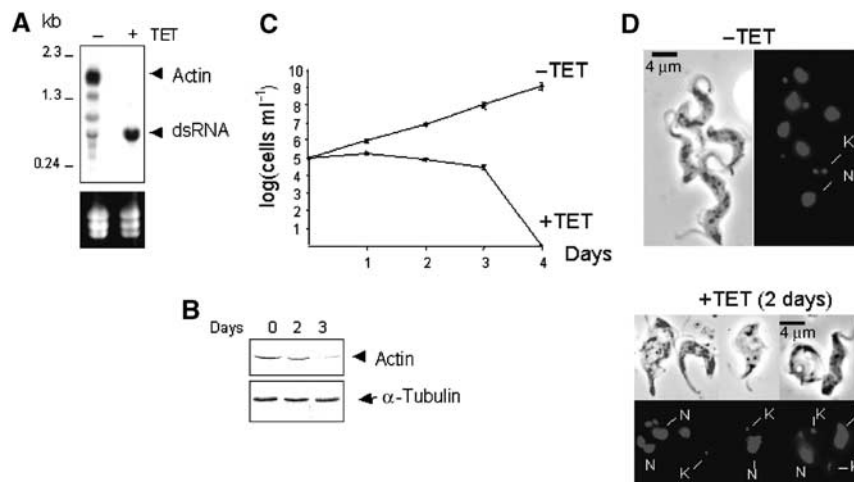
Conditional ablation of mRNAs through RNAi has become a powerful method for investigating gene function in trypanosomes (Ngo *et al*, 1998; Bastin *et al*, 2000; Bringaud *et al*, 2000; LaCount *et al*, 2000; Shi *et al*, 2000; Wang *et al*, 2000). We employed a construct that allowed the tetracycline-inducible production of a double-stranded RNA (dsRNA) containing the first 493 nucleotides of the actin open reading frame. Ablation of the *actin* mRNA had a dramatic and rapid effect on the growth of bloodstream forms (Figure 3A–C). The transcript was no longer detectable, and cell division ceased within 24 h of induction of the dsRNA (Figure 3A and C). The decrease in actin expression was less dramatic, and significant changes in the level of the protein were observed only after 2–3 days induction (Figure 3B). Throughout this period, there was no change in the level of expression of tubulin. Although cell division quickly ceased after induction of RNAi, the cells remained intact for up to 3 days. However, during this period, the cell morphology changed dramatically. While there was heterogeneity in the population, the cells became increasingly enlarged or swollen at the posterior end of the cell (Figure 3D). Examination of nonfixed, living cells using



**Figure 1** Characterization of actin in bloodstream and procyclic forms of *T. brucei*. (A) Western blot analysis of extracts from whole cells ( $5 \times 10^6$  cells lane<sup>-1</sup>) using specific antibodies against actin. The lanes correspond to the following life-cycle forms: SL, bloodstream slender forms; ST, bloodstream stumpy forms; P, procyclic forms. The lower section of the panel presents the level of  $\alpha$ -tubulin as a control for loading. (B) Localization of actin in procyclic forms (a) and bloodstream slender forms (b). The DAPI staining reveals the position of the nucleus (N) and kinetoplast (K).



**Figure 2** Actin colocalizes with the endocytic pathway in bloodstream forms. (A) Colocalization with tomato lectin (TL-FITC). (B) Colocalization with the CB1 epitope of the p67 glycoprotein. DAPI fluorescence (blue) reveals the position of the nucleus (N) and kinetoplast (K).



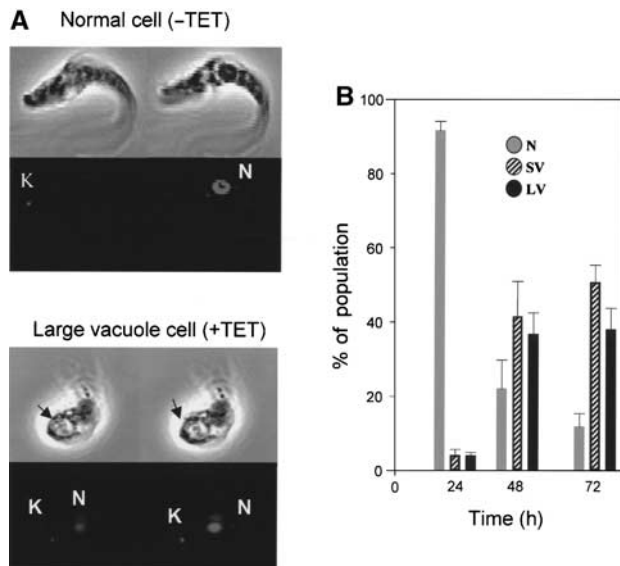
**Figure 3** Effect of actin RNAi on bloodstream forms. (A) Northern blot analysis of 15 μg of total RNA from actin RNAi cells incubated in the presence (+) or absence (-) of tetracycline, using the actin probe for hybridization. The lower part of the panel presents the ethidium bromide staining of the RNA as a control for loading. (B) Western blot analysis of total cell extracts ( $5 \times 10^6$  cell equivalents lane<sup>-1</sup>) from RNAi cells incubated for different periods with tetracycline, using anti-actin antibodies and anti- $\alpha$ -tubulin antibodies. (C) Growth of bloodstream RNAi cells incubated in the presence (+) or absence (-) of tetracycline. Error bars represent the mean  $\pm$  s.e.m. of four determinations. Where no error is shown, the error was less than the representation of the point. (D) Effect of actin RNAi on the cellular morphology of fixed cells after 2 days induction with tetracycline. DAPI fluorescence reveals the position of the nucleus (N) and kinetoplast (K).

confocal microscopy during the course of the induction clearly demonstrated the presence of a growing, phase light vesicle or vacuole located between the kinetoplast and nucleus (Figure 4A). The morphological composition of the population was analyzed during a 3-day induction (Figure 4B). Three distinct cell morphologies were discernable: normal cells (N), cells with a small vacuole in the posterior end (SV) and cells where the large phase light vacuole was clearly visible and occupied most of the cell (LV). Presumably, the SV cells were the precursors of the LV cells. The latter cells were readily recognizable and were visually identical to the 'BigEye' phenotype recently reported in clathrin heavy-chain RNAi cells (Allen *et al*, 2003). After

24 h induction, most of the cells exhibited a normal morphology and only about 10% of the population possessed SV/LV morphologies. However, during the next 48 h, the situation was reversed. After 3 days of induction, almost all cells exhibited an aberrant morphology and shortly afterwards they began to lyse.

#### **Loss of actin blocks the formation of coated vesicles from the flagellar pocket in bloodstream forms**

The specific colocalization of actin with the endocytic pathway, as well as its requirement for growth, raised the possibility that actin might be directly involved in endocytosis in bloodstream forms. This view is consistent with emerging



**Figure 4** Analysis of cellular morphologies in bloodstream actin RNAi cells. (A) Confocal sections through a living cell after 3 days of actin RNAi. The panel presents two serial sections containing phase contrast (upper) and fluorescence (lower) images captured using a Leica TCS SP2 confocal microscope. The nucleus (N) and kinetoplast (K) were revealed by staining with ethidium bromide. Note the presence of a large vacuole (arrow) in the sections located between the nucleus (N) and kinetoplast (K) consistent with enlargement of the flagellar pocket in cells incubated in the presence of tetracycline. Interference haloes in the phase images are due to cell motility. (B) Relative proportion of three distinct cell morphologies during a 3-day induction of actin RNAi. Samples of cells were counted six times and the different cell morphologies were expressed as a relative percentage of the total number of cells in the population. Results are presented as the mean  $\pm$  s.e.m. of four determinations. N, normal cells; SV, cells with a small vacuole in the posterior end; LV, cells where the large phase light vacuole was clearly visible and occupied most of the cell.

ideas on links between endocytosis and the actin cytoskeleton in other eukaryotes (Lamaze *et al*, 1997; Geli and Riezman, 1998; Qualmann *et al*, 2000; McPherson, 2002). The typical distribution of tomato lectin fluorescence in a normal bloodstream slender form, which starts at the flagellar pocket located close to the kinetoplast and extends in an anterior direction towards the nucleus, was no longer observed in cells subjected to actin RNAi (Figure 5A). In these cells, binding was now limited to a single, intense spot located close to the kinetoplast, which suggested that tomato lectin no longer stained endosomal and lysosomal compartments but was restricted to the flagellar pocket only. The nucleus and kinetoplast did not appear to be affected by the loss of actin. Electron microscopy revealed significant changes in the structure of the endocytic pathway. An obvious feature in some sections was the almost complete absence of the formation and fission of coated vesicles from the flagellar pocket membrane, which at this stage appeared normal in size (Figure 5B). However, the lumen of the pocket appeared to contain more tomato lectin binding sites as indicated by the more numerous gold particles in the actin RNAi cells compared to control cells. Significantly, in many sections taken after 48 h induction, the flagellar pocket was grossly enlarged as indicated by the relative size of the flagellum compared to the lumen of the pocket (Figure 5B).

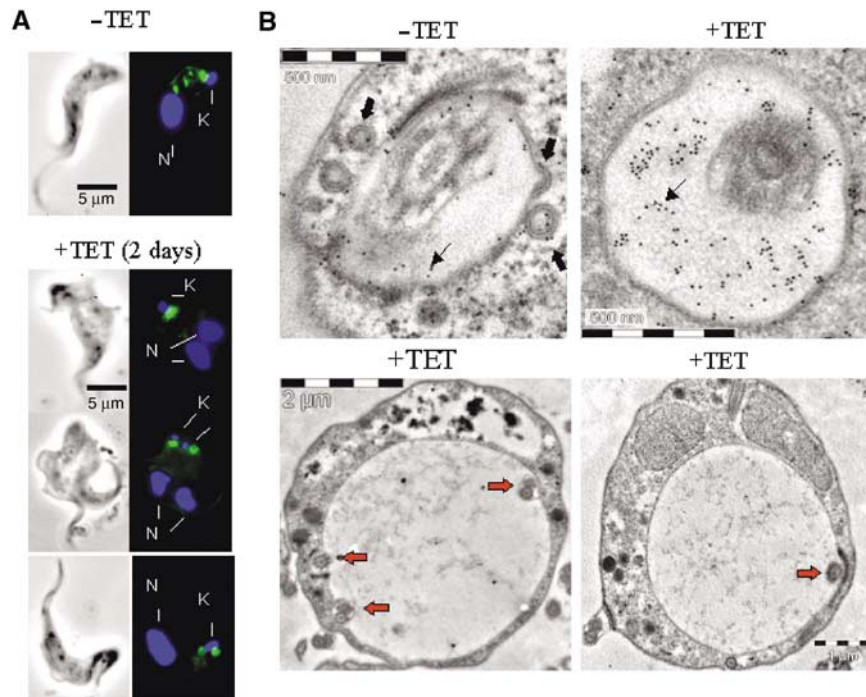
Multiple flagella were also observed in some sections of the enlarged flagellar pocket. This finding is consistent with a failure to complete the process of cell division in cells undergoing actin RNAi, since one of the earliest morphological events in cell division in trypanosomes is the formation of a new flagellum (Gull, 1999). The enlargement of the flagellar pocket observed in electron micrographs correlated well with the presence of the enlarged phase light vacuole located between the kinetoplast and nucleus in confocal sections of living cells (Figure 4A). These data support the view that actin is required for the formation and trafficking of vesicles from the flagellar pocket in bloodstream forms of *T. brucei*.

#### Functional effects of the loss of actin in bloodstream forms

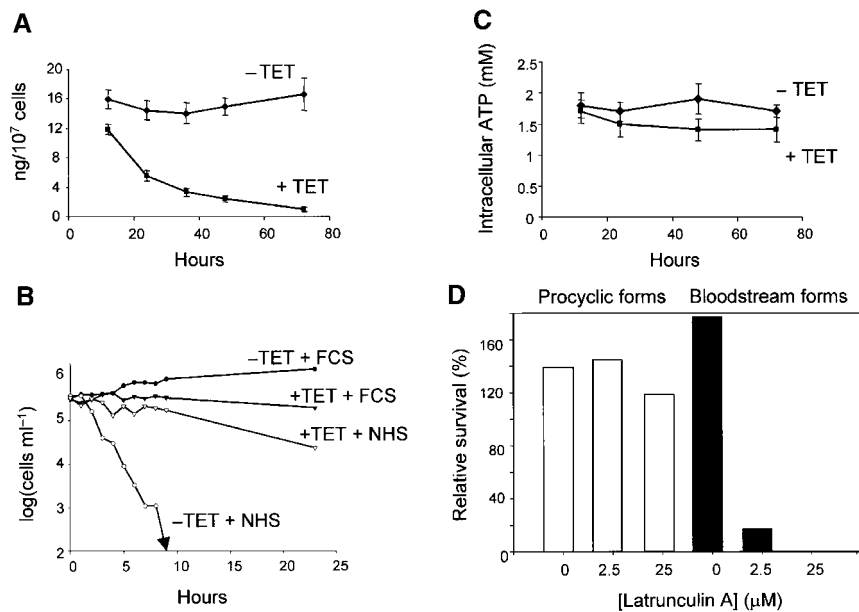
As vesicular traffic from the flagellar pocket appears to be the only route for the uptake of essential macromolecules, the effect of the loss of actin on endocytic activity was investigated. Direct measurement of transferrin uptake by cells subjected to actin RNAi demonstrated that within 24 h of induction, ligand uptake was less than half that observed for control cells, and within 48 h it was effectively abolished (Figure 6A). The effect of human serum on *T. brucei* is another parameter that can be used to monitor endocytic activity. Human but not foetal calf serum lyses these trypanosomes and lysis requires endocytosis and delivery of human apolipoprotein L-I to the lysosome (Vanhamme *et al*, 2003). Trypanosomes subjected to actin RNAi were significantly more resistant to lysis by human serum and a substantial number remained even after 8 h incubation in the presence of human serum, whereas the control cells were completely lysed (Figure 6B). Thus, endocytosis of apolipoprotein L-I appeared to require actin. The inhibition of endocytosis by actin RNAi was not related to secondary metabolic effects since the ATP levels in actin RNAi cells were comparable to those of control cells (Figure 6C). Together, these results support the view that endocytic activity in bloodstream forms is compromised when actin expression is repressed. This conclusion was consistent with the effect of latrunculin A on the growth of bloodstream compared to procyclic trypanosomes, where endocytosis is thought to be less important. Latrunculin A is a cell-permeant actin antagonist that binds to monomeric G-actin and has been used to investigate the effect of F-actin disassembly on several physiological functions (Coue *et al*, 1987; Spector *et al*, 1989). Significantly, latrunculin A was toxic for bloodstream but not procyclic trypanosomes. Only 10% of bloodstream forms remained after an incubation of 8 h with 2.5  $\mu$ M latrunculin A, while no cells survived when incubated at a 10-fold higher concentration of the drug. However, the same concentrations of latrunculin A had no effect on procyclic cells, which continued to grow in the presence of latrunculin A (Figure 6D). Longer incubations (24 h) revealed that the doubling time of procyclic cells was the same in the presence and absence of these concentrations of latrunculin A (data not shown).

#### Loss of actin is not lethal in procyclic forms

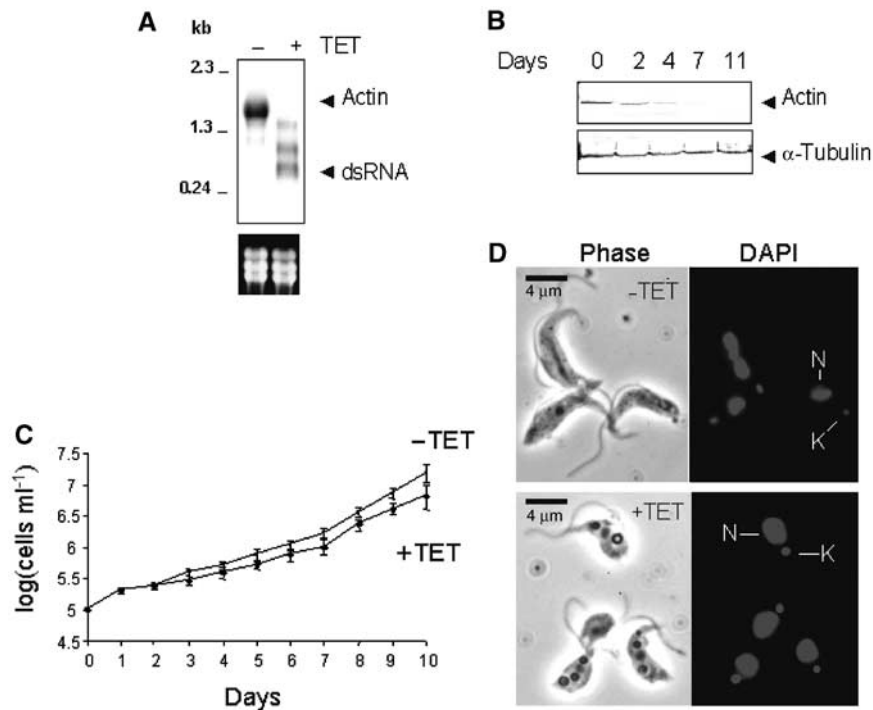
As observed for bloodstream forms, the *actin* mRNA was lost within 24 h of induction of actin RNAi in procyclic forms (Figure 7A). Western blotting demonstrated that the loss of actin was progressive in these cells and up to 7 days



**Figure 5** Effect of actin RNAi on the endocytic pathway of bloodstream forms. (A) Location of tomato lectin binding sites using FITC-TL (green). DAPI fluorescence (blue) reveals the position of the nucleus (N) and kinetoplast (K). (B) Loss of vesicles budding from the flagellar pocket membrane (top panels, solid arrowheads). The 10 nM colloidal gold particles (arrows) reveal the presence of tomato lectin binding sites, which appear to be more abundant in the actin RNAi cells (+TET). The lower panels reveal enlargement of the flagellar pocket in actin RNAi cells. Note the size of the lumen of the pocket with respect to the flagellum (red arrowheads) and size bar. In some sections, multiple flagella are visible.



**Figure 6** Actin RNAi leads to a loss of endocytic activity in bloodstream forms. (A) Effect of actin RNAi on transferrin uptake. The uptake of  $^{125}\text{I}$ -labelled transferrin by cells, incubated in the presence (■) or absence (●) of tetracycline for various times, was measured as described in the Experimental procedures. The results are expressed as the mean  $\pm$  s.e.m. of four determinations. (B) Actin RNAi inhibits lysis of trypanosomes by human serum. Bloodstream forms were subjected to actin RNAi by incubation in the presence or absence of tetracycline for 48 h. These cells were subsequently incubated in HMI9 medium supplemented with human (NHS) or nonlytic foetal calf serum (FCS) both at a final concentration of 30%. (C) Effect of actin RNAi on cellular ATP levels. Bloodstream forms were subjected to actin RNAi by incubation in the presence (■) or absence (●) of tetracycline. Samples were removed at various times, and the intracellular ATP level was determined as described previously (Nolan and Voorheis, 1992). The results are expressed as the mean  $\pm$  s.e.m. of four determinations. (D) Effect of latrunculin A on the growth of bloodstream and procyclic forms of *T. brucei*. Bloodstream or procyclic forms were incubated in the presence or absence of latrunculin A. After 8 h, the cells were counted and expressed as a percentage of the number of cells present at the beginning of the incubation. There were no live bloodstream forms remaining when 25  $\mu\text{M}$  latrunculin was employed.



**Figure 7** Effect of actin RNAi on procyclic forms. (A) Northern blot analysis of 15  $\mu$ g of total RNA from actin RNAi cells incubated in the presence (+) or absence (-) of tetracycline, using the actin probe for hybridization. The lower part of the panel presents the ethidium bromide staining of the RNA as a control for loading. (B) Western blot analysis of total cell extracts ( $5 \times 10^6$  cell equivalents lane<sup>-1</sup>) from RNAi cells incubated for different periods with tetracycline, using anti-actin antibodies and anti- $\alpha$ -tubulin antibodies. (C) Growth of procyclic RNAi cells incubated in the presence (+) or absence (-) of tetracycline. Error bars represent the mean  $\pm$  s.e.m. of four determinations. Where no error is shown, the error was less than the representation of the point. (D) Effect of actin RNAi on the cellular morphology after 2 days induction with tetracycline. DAPI fluorescence reveals the position of the nucleus (N) and kinetoplast (K).

induction was required before the protein was no longer detectable (Figure 7B). Despite the loss of actin, these cells continued to grow and divide for many generations at a rate similar to that of noninduced cells (Figure 7C). This result was in agreement with a previous study that demonstrated that a 10- to 20-fold downregulation of *actin* mRNA had no deleterious effects on the sustained growth of procyclic forms in culture (Shi *et al*, 2000). Although the loss of actin did not affect cell division or motility, procyclic cells that lacked actin were morphologically different from control cells. These cells appeared to be smaller and stumpy in appearance, but both the nucleus and kinetoplast appeared to be normal in size and position (Figure 7D).

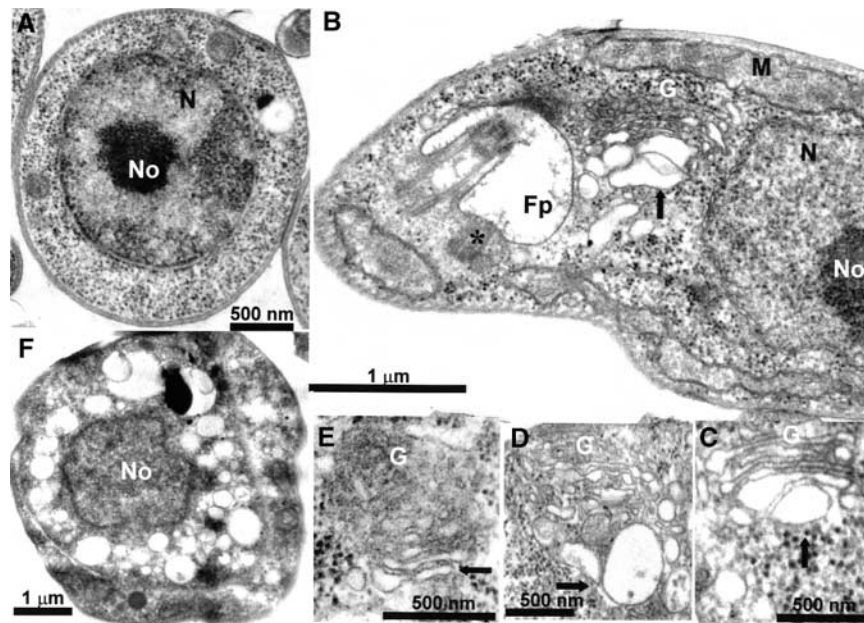
Procyclic forms subjected to actin RNAi for 8 days were examined by electron microscopy to assess possible ultrastructural effects of actin loss (Figure 8). The loss of actin did not appear to affect most structures. The nucleus, nucleolus, nuclear and plasma membranes, the kinetoplast, the flagellum and mitochondrion all appeared to exhibit a normal morphology. However, there was a defect in the structure of the Golgi complex (Figure 8B). This defect was most obvious in the *trans* region of the Golgi where the cisternae and membrane stacks became distorted and enlarged (compare Figure 8C–E). These deformed stacks appeared to give rise to a population of electron-lucent vesicles located close by. In some sections, these vesicles, with diameters of 40–500 nm, were seen to surround the nucleus completely (Figure 8F). Significantly, the flagellar pocket appeared normal in size and morphology, and the presence of a new basal body/flagellum complex close to the pocket indicated that cell division

continues in actin RNAi cells even when the structure of the Golgi complex was abnormal (Figure 8B).

## Discussion

Actin is a conserved and ubiquitous protein considered to be essential in eukaryotes because of its involvement in movement, morphology and trafficking. This study presents the first detailed characterization of actin in any parasitic protozoan and several new findings have emerged. Although actin is expressed at similar levels through the life cycle of *T. brucei*, the protein does not appear to be essential for the growth of procyclic forms, at least in culture. This result is in agreement with the finding of Shi *et al* (2000) that a 10- to 20-fold downregulation of actin mRNA had no effect on the growth and viability of cells. However, these workers were unable to evaluate the effect of this decrease on actin expression and suggested that residual amounts of actin that would be sufficient to support growth might remain. We found that procyclic cells continued to grow and divide at a normal rate even when actin was no longer detectable. This surprising result suggests that actin is either truly dispensable in procyclic cells or has some function not required under culture conditions. Nevertheless, there were apparently benign effects on cell shape, which indicated that actin might have some nonessential role in cellular architecture. A possible clue to this role was indicated by electron microscopy, which demonstrated a defect in the structure of the Golgi, which was especially noticeable at the *trans* face of the organelle where the cisternae and stacks became distorted





**Figure 8** Loss of actin in procyclic cells appears to lead to a defect in the structure of the Golgi complex. (A) Section through the nucleus of control procyclic cells incubated in the absence of tetracycline. (B) Disruption of the Golgi complex (G) was especially obvious in the region of the *trans*-Golgi where the cisternae and stacks (block arrow) became distorted and enlarged in procyclic cells devoid of actin. These deformed stacks appeared to give rise to a population of electron-lucent vesicles. Note the presence of an additional basal body/flagellum complex (\*) in a cell with a distorted Golgi, indicating that cell division was proceeding in this cell. (C, D) Details of the Golgi region of procyclic actin RNAi cells showing distortions to the *trans*-Golgi. (E) The complex in control cells exhibits a normal structure and the *trans* region is not deformed. (F) In some actin RNAi cells, multiple vesicles were seen to surround the nucleus and these vesicles were not present in control cells (compare with (A)). The loss of actin did not appear to affect other structures, such as the flagellar pocket (Fp), nucleus (N), nucleolus (No), nuclear or plasma membranes, the kinetoplast (K) and mitochondrion (M), which appeared to exhibit a normal morphology (B).

and enlarged. These stacks appeared to give rise to a population of heterogeneous vesicles that accumulated in the region and around the nucleus. In view of these results, it is tempting to speculate that the loss of actin somehow impairs vesicle traffic from the *trans*-Golgi but that this disruption is not lethal. It is striking that the loss of actin appears to have such a specific structural defect given that the protein appears to be distributed throughout the cell. A major disruption of the *trans*-Golgi region was also observed in procyclic cells depleted in the clathrin heavy chain (Allen *et al*, 2003). These authors suggested that loss of clathrin caused a severe defect in intracellular vesicle transport. While similar in some respects, it is important to note that the clathrin and actin RNAi phenotypes were not identical in procyclic cells. For example, the number of vesicles appeared to be greater and their distribution more widespread throughout the cytoplasm in clathrin RNAi than in actin RNAi cells. In addition, the loss of actin did not result in the round phenotype observed in the case of the clathrin, which was attributed to a build-up of internal pressure due to the presence of excess membrane accumulating in the cytoplasm. Of course, the most striking difference between the two situations is that loss of the clathrin heavy chain is lethal, whereas loss of actin is not. Remarkably, the clathrin heavy chain, which is significantly downregulated in procyclic cells, is essential (Morgan *et al*, 2001), while actin, which is constitutively expressed, is not.

While actin may not be required in procyclic cells, it is clearly an essential protein in bloodstream forms. These cells stopped growing very soon after the induction of double-stranded *actin* RNA, and within 24 h the mRNA was no longer detectable. Interestingly, the effect on growth occurred prior

to significant changes in the level of actin, suggesting that bloodstream trypanosomes are very sensitive to the amount of actin present and that cell division requires the continuous expression of the protein. Another interesting finding was the redistribution of actin during the life cycle of the trypanosome. Actin was present throughout procyclic cells, as observed in many eukaryotes (Small *et al*, 1999), but in bloodstream forms actin clearly exhibited a very polarized distribution and colocalized with the endocytic pathway. Consistent with this location, the use of RNAi demonstrated that both endocytosis of transferrin and uptake of apolipoprotein L-I from human serum were inhibited when actin expression was repressed in bloodstream forms.

There is general agreement that actin plays a role in endocytosis in eukaryotes but precisely at what steps in the process remains unclear. Endocytosis involves distinct steps that include membrane invagination, coated vesicle formation, detachment of the newly formed vesicle and movement of this vesicle away from the plasma membrane, and in theory any or all of these steps might involve the actin cytoskeleton (Qualmann *et al*, 2000; Jeng and Welch, 2001). However, in the case of bloodstream trypanosomes, it would appear that, at a minimum, actin is required for the formation of coated vesicles from the flagellar pocket membrane. This conclusion, which does not preclude additional involvement at later steps, is based on several findings. First, depletion of actin was accompanied by a loss of endocytic activity. Second, tomato lectin binding was restricted to a single spot that appeared to correspond to the flagellar pocket in actin RNAi cells. Immunogold electron microscopy also suggested an accumulation of tomato lectin binding sites in the

flagellar pocket in these cells. These observations are consistent with evidence indicating that the flagellar pocket is a conduit for the routing of proteins, for example, ESAG6/7/CB1/p67 or ISG<sub>100</sub>, which contain PNAL and bind to tomato lectin, from the Golgi to the endosomal/lysosomal compartments (Brickman and Balber, 1993, 1994; Nolan *et al*, 1997, 1999). Presumably, loss of traffic from the pocket leads to an accumulation of these proteins in the flagellar pocket. Third, ultrastructural analysis revealed that the formation and budding of vesicles from the flagellar pocket membrane was less in actin RNAi cells. Finally, the loss of actin was accompanied by a massive enlargement of the flagellar pocket identical to that observed when clathrin was depleted (Allen *et al*, 2003). These data all indicate that actin is required for the formation of clathrin-coated vesicles from the flagellar pocket membrane but not for traffic to the flagellar pocket. Although we have not demonstrated directly that exocytosis was not affected, the increased tomato lectin binding in the flagellar pocket in actin RNAi cells was consistent with continued delivery of glycoproteins that bind to tomato lectin to the pocket. The combination of loss of vesicle traffic from the flagellar pocket along with continued vesicle delivery to the pocket would lead to an increase in the area of the pocket membrane, which in turn is forced to invaginate into and occupy an increasing volume of the cytoplasm because the subpellicular network of microtubules fixes the area of the pellical surface (Vickerman and Preston, 1976; Kohl and Gull, 1998). These same processes lead to the massively enlarged pocket, termed the 'BigEye' phenotype, observed during the ablation of the clathrin heavy chain in bloodstream forms of *T. brucei* (Allen *et al*, 2003). Interestingly, the curve of the enlarged flagellar pocket membrane was as smooth in actin RNAi as in 'BigEye' cells, suggesting that loss of actin caused an equally potent block in vesicular traffic as clathrin. Although actin is required for the first step in the endocytic process, the presence of actin throughout the pathway suggests that the actin cytoskeleton might also be involved at later steps.

As bloodstream trypanosomes are dependent on endocytosis of growth factors, it is clear why actin is an essential protein during this stage of the life cycle. However, if endocytosis occurs in procyclic cells, either this process is not essential for growth or it does not involve the actin cytoskeleton. Significantly, loss of actin or clathrin does not appear to affect the morphology of the flagellar pocket in procyclic cells. The differential inhibitory effect of latrunculin on the growth of bloodstream and procyclic forms also supports this view and suggests that the assembly of F-actin is the key step. If this is the case, then it seems reasonable that the actin nucleation machinery is also likely to be involved in endocytosis in bloodstream forms. Although we have identified several homologues of the Arp2/3 complex in the trypanosome genome database, so far none of the typical nucleation-promoting factors appear to be present (Welch and Mullins, 2002).

## Materials and methods

### Trypanosomes

Procyclic *T. brucei* strains EATRO 1125 and 29-13 and bloodstream 427, strain 90-13 were grown in SDM-79 (Brun and Schonenberger, 1979) and HMI9 medium (Hirumi and Hirumi, 1989), respectively, and supplemented with 15% foetal bovine serum. The pleomorphic *T. brucei* variant clone AnTat 1.1 was grown in mice. Procyclic 29-13 and bloodstream forms, strain 90-13, which both harbour

integrated genes for T7 polymerase and tetracycline repressor (Wirtz *et al*, 1999), were used for RNAi. Transfection of both bloodstream and procyclic forms was performed as described previously (Wirtz *et al*, 1998) using 10 µg of *NotI*-linearized DNA electroporated into 400 µl of  $2 \times 10^7$  cells. Transfected cells were incubated overnight in 24 ml of HMI9 at 37°C, 5% CO<sub>2</sub> (bloodstream forms) or 10 ml of SDM79 medium at 27°C (procyclic forms) before being diluted to  $1 \times 10^5$  cells ml<sup>-1</sup> and subjected to drug selection in 24-well plates. The transfectants were selected with 2.5 µg ml<sup>-1</sup> phleomycin (Wang *et al*, 2000).

### Plasmid constructions and expression of recombinant proteins

The Tbactin RNAi construct was made using the vector pZJM (Wang *et al*, 2000) to allow tetracycline-inducible expression of dsRNA from a T7 promoter in *T. brucei* 427, strains 90-13 and 29-13 (Wirtz *et al*, 1999). A fragment corresponding to the first 493 nucleotides of the actin open reading frame was amplified by PCR and cloned into the *HindIII* and *XhoI* sites of pZJM to yield pZJMactin. A comparison of the coding sequence we employed for actin RNAi with sequences in the current databases revealed that the highest level of identity was with a sequence that encoded a putative actin-related protein and the overall identity was 40%. A recent study on RNAi in *T. brucei* has shown that expression of *PAR2* dsRNA, which has 83% identity with *PFRA* including nine blocks of >20 nt total identity, did not produce a significant reduction in the total amount of *PFRA* RNA or *PFRA* protein (Duran-Dubief *et al*, 2003). We have also shown that RNAi of a trypanosome *SIR2* mRNA (*TbSIR2RP1*) did not affect the expression of two other *SIR2*-related genes that share approximately 47% sequence identity with the region employed for the *TbSIR2RP1* construct (García-Salcedo *et al*, 2003). In view of these results, it seems reasonable to assume that the *actin* dsRNA produced in this study would be unlikely to affect by cross RNAi the actin-related sequences currently in the databases. Plasmid pGEX-Tbactin was generated by cloning the entire actin coding sequence in frame with the C-terminus of GST into the *BamHI/XhoI* site of pGEX5. Expression of the recombinant proteins was induced in mid-logarithmic phase DH5α cells, by incubation with 0.1 mM isopropyl β-D-thiogalactopyranoside for 5 h at 37°C. The bacteria were lysed by sonication and the fusion proteins were purified on glutathione-agarose by standard procedures (Amersham-Pharmacia). Anti-actin antibodies were raised in rabbits against the GST-actin fusion polypeptide.

### Immunofluorescence microscopy

Trypanosomes were washed once with PBS (NaCl, 136 mM; KCl, 3 mM; Na<sub>2</sub>HPO<sub>4</sub>, 16 mM; KH<sub>2</sub>PO<sub>4</sub>, 3 mM; sucrose 40 mM; glucose, 10 mM; pH 7.6) and then resuspended ( $2 \times 10^7$  cells ml<sup>-1</sup>) in the same buffer at 0–4°C. The cell suspension was mixed gently by inversion several times with an equal volume (20–25°C) of freshly prepared paraformaldehyde (6%, w/v) in PBS adjusted to pH 7.6. The suspension was incubated at room temperature for 10 min. After washing, the fixed cells were resuspended at  $2 \times 10^7$  cells ml<sup>-1</sup> and applied to polylysine-coated slides. After attachment of the cells, the slides were washed with PBS containing glycine (1 mg ml<sup>-1</sup>) and permeabilized in 0.1% Triton X-100 in PBS and processed as described previously (García-Salcedo *et al*, 2002) using anti-Tbactin antibodies (1/500 dilution) or CB1 monoclonal antibody (1/250 dilution). Fluorescein isothiocyanate-labelled or Texas red-labelled anti-mouse IgG secondary antibodies were used at 1/100 dilution. TL-FITC conjugated was used at 1/250 dilution as previously described (Nolan *et al*, 1999). Images were taken on a Zeiss Axioplan microscope coupled to a CCD camera.

### Electron microscopy immunocytochemistry

Bloodstream or procyclic trypanosomes transfected with pZJMactin were incubated, for 48 h or 8 days respectively, in the presence or absence of tetracycline (1 µg ml<sup>-1</sup>) and then fixed for 15 min at room temperature in 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Cell pellets, wrapped in 1% agarose, were dehydrated with increasing concentrations of methanol and embedded in Lowicryl K4M at –20°C. Polymerization was performed for 4 days at –20°C under low-wavelength UV light. Ultrathin sections were collected in formvar-coated nickel grids and floated on drops of 1% BSA in PBS buffer for 30 min at room temperature, and incubated overnight at 4°C on biotinylated tomato



lectin (50 µg ml<sup>-1</sup>) in PBS/1% BSA. After washing, grids were incubated overnight at 4°C with goat anti-biotin antibodies (1/100 dilution) conjugated to 10 nM colloidal gold particles (British BioCell) and then stained with uranyl acetate and lead citrate. Observations were made on a Tecnai 10 electron microscope. The images were captured with a MegaView II camera and processed with AnalySIS (GmbH) and Adobe Photoshop software.

### Transferrin uptake

Bovine transferrin (Sigma) was labelled with <sup>125</sup>I using the iodogen method essentially as described previously (Salmon *et al*, 1994). Trypanosome cell lines transfected with the inducible actin RNAi construct were grown to a density of approximately 1.0 × 10<sup>6</sup> ml<sup>-1</sup> in HMI9 medium containing the appropriate selection antibiotics prior to the addition of tetracycline (1 µg ml<sup>-1</sup>). Cells were removed from culture at various times after the addition of tetracycline and washed in serum-free RPMI medium (4°C) containing 5 mg ml<sup>-1</sup> ovalbumin, 0.15 mM adenosine and 30 mM Hepes (pH 7.4). After the washing steps, the cells were counted and resuspended at

1 × 10<sup>7</sup> ml<sup>-1</sup> in the same medium and incubated at 37°C (CO<sub>2</sub>, 5.5%) for 20 min prior to the addition of 15 µg ml<sup>-1</sup> labelled transferrin. After 60 min, samples of 1 ml were removed and added to 10 ml of ice-cold incubation medium containing 5% foetal bovine serum and 5 × 10<sup>6</sup> ml<sup>-1</sup> wild-type bloodstream form, to allow the formation of a stable pellet of cells during the serial washing steps prior to analysis of the cellular content of <sup>125</sup>I-labelled transferrin by gamma counting.

### Acknowledgements

We thank Dr Paul Englund (Johns Hopkins Medical School, Baltimore) for providing us with constructs and cell lines to perform the RNAi. We also thank K Woods for technical support. This work was supported by the Wellcome Trust through the award of a Senior Fellowship to DPN and the Interuniversity Poles of Attraction Programme of the Belgian State Prime Minister's Office—the Federal Office for Scientific, Technical and Cultural Affairs.

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