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ORIGINAL PAPER

Anisomorphic Cell Division by African Trypanosomes

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In the bloodstream of a mammalian host, African trypanosomes are pleomorphic; the shorter, non-proliferative, stumpy forms arise from longer, proliferative, slender forms with differentiation occurring via a range of morphological intermediates. In order to investigate how the onset of morphological change is co-ordinated with exit from the cell cycle we first characterized slender form cell division. Outgrowth of the new flagellum was found to occur at a linear rate, so by using outgrowth of the new flagellum as a temporal marker of the cell cycle we were able determine the order in which single copy organelles (nucleus, kinetoplast and mitochondrion) were segregated. We also found that flagellar length was an effective marker of the slender to stumpy differentiation and were, therefore, able to study both cell division and differentiation. When these differentiating cells were compared to cells undergoing proliferative cell division, they were found to be anisomorphic – showing discernible differences not only in the length of their new flagella but also in the shape and size of the cells and their nuclei.

Introduction

African trypanosomes transmitted by the bite of the tsetse fly are responsible for serious bovine (ngana) and human (sleeping sickness) disease. In recent years, the incidence of African trypanosomiasis has increased to levels not seen since the 1930s (World Health Organization 1996). In regions such as the Democratic Republic of Congo, sleeping sickness is once again considered epidemic and kills more people than AIDS (World Health Organization 1996). African trypanosomes have a complex life cycle that alternates between proliferative and nonproliferative life cycle stages (Vickerman 1985). The proliferative form that perpetuates disease in the chronically infected mammalian host is the slender form. At high parasitaemia, slender forms give rise to the non-proliferative stumpy forms, which are preadapted for survival in the tsetse midgut. In an immunocompetent mammalian host, stumpy forms are generally cleared from the circulation by the immune response after a few days (Balber 1972; Turner et al. 1995). If all slender forms underwent differentiation, then the infection would be cleared after a single wave of parasitaemia. This does not occur, however, because differentiation does not occur in the whole population

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Abbreviations: IFA: immunofluorescence assay; DHLADH: dihydrolipoamide dehydrogenase; TAO: trypanosome alternative oxidase; PFR: paraflagellar rod; DAPI: 4',6-diamidino-2-phenylindole dichydrochloride.

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and because the slender forms remaining undergo a high rate of antigenic variation (Turner 1999). Consequently, slender variants are able to renew the infection as most of the stumpy forms are cleared and this can lead to the characteristic undulating parasitaemia that is sometimes observed.

The trypanosome cell has several features that make it a valuable model for studying organellar biogenesis and segregation in preparation for cell division (Gull 1999; Seebeck et al. 1988). The trypanosome cell possesses large single copy organelles: one flagellum subtended by a basal body and a probasal body, one nucleus, one mitochondrion and a unitary mitochondrial genome – the kinetoplast. These structures must be replicated and segregated with fidelity once per cell cycle, however, unlike other eukaryotes, trypanosomes have no detectable actin cytoskeleton and so are largely reliant on microtubule mediated processes to achieve cell division.

In considering trypanosome cell division as a potential target for therapeutics, it is clearly preferable to study the pathogenic, slender bloodstream form. Historically, however, it was only possible to culture the non-infective insect midgut (procyclic) form. This led to most studies on trypanosome cell division being undertaken on the procyclic form. It is clear, however, that key elements of cell division such as the length of the cell cycle may vary between the slender and procyclic form (Turner et al. 1995). The forms are also structurally and morphologically different, which necessitates differences in spatial arrangements of organelles during the cell division of the different forms. Notably, the slender form also has a simple linear mitochondrion – compared with the complex branching mitochondrion of the procyclic form (Tyler et al. 1997; Vickerman 1965). In fact, this means that the manner in which mitochondrial segregation occurs is more readily observed in the slender form. Consequently, in preparing a description of cell division in the slender form, we have also detailed the process of mitochondrial segregation. Previous studies, focussed on procyclic cell division, found that cell division was essentially proliferative and homogenous during the logarithmic phase of growth (Robinson et al. 1995; Sherwin et al. 1989). It has been shown in vitro, that differentiation to the stumpy form occurs in a density dependent fashion with high efficiency and rapid kinetics (Vassella et al. 1997). Working in vivo with a murine model, we have previously shown that division of bloodstream forms is not solely proliferative, and that non-dividing stumpy forms can arise by a heterologous differentiation-division (Tyler et al. 1997). Here, we describe anisomorphic variation between the proliferative and differentiation pathways of cell division and use these morphological differences to elucidate further the manner in which the slender to stumpy differentiation occurs.

Results

The Rate of New Flagellum Outgrowth is Constant

In both slender and procyclic form trypanosomes, one of the first detectable events indicating commitment to a new round of cell division is maturation of the probasal body enabling it to subtend the new flagellum (Sherwin et al. 1987; Tyler 1998). Thereafter, it is believed that the flagellum grows throughout much of the cell cycle until cytokinesis. This has allowed the length of the new flagellum to be used as a marker of progress through cell division (Robinson 1995). However, it is not readily feasible to follow flagellar growth in either a single cell or in synchronized culture and it has not previously been determined whether the rate of flagellar elongation is constant during this period. We reasoned that by randomly sampling cells with two flagella, measuring the new flagellar lengths and then sequentially arranging them by length of the new flagellum, that we would obtain a gradient which would speak to the rate of flagellar elongation. In the population analyzed, 54% of cells had two flagella (based on a count of 1000 cells). We sampled 100 such cells and measured both new and old flagellar length. We then sorted them by the length of their new flagella (Fig. 1). The lengths of new flagella all fell between 1 and 27 μm and the gradient obtained was essentially linear.

This result indicated that for the period the new flagellum grows before cytokinesis, growth is likely to occur at an approximately constant rate at least within the resolution of our assay. The mean doubling time for this trypanosome line in vivo is approximately 6.3 hours in the bloodstream form (Tyler et al. 2001). So we can make a crude estimate for the rate of flagellar elongation in the bloodstream form to be 8 μm per hour ($0.54 \times 6.3 \div 27$). This estimate assumes the mean doubling time to be the true cell cycle length and that the whole population is undergoing a proliferative cell cycle of equal length. In all likelihood these approximations are not true and some cells are not fully engaged in a proliferative cell cycle and so 8 μm per hour is probably a slight overestimate (See Woodward and Gull 1990 for detailed discussion). Nevertheless, the rate of flagellar elongation in the bloodstream form is clearly considerably faster than a recent estimate of 3.6 μm per hour for the procyclic form (Bastin et al. 1999). A result consistent with the mean flagellar length of bloodstream forms being longer than that of procyclic forms while their cell cycle is shorter.

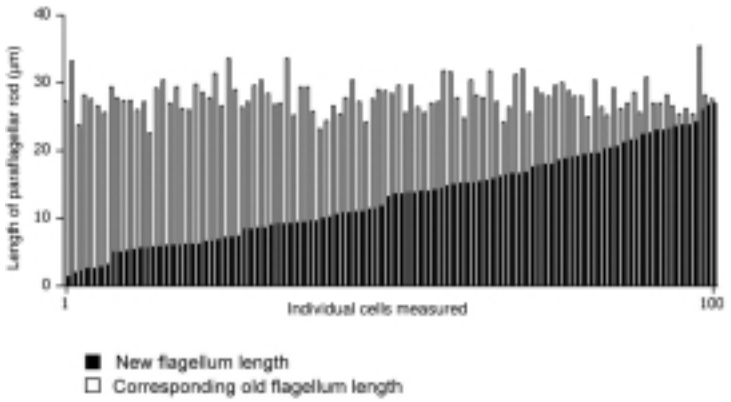

Figure 1. In cells with two flagella, new flagella (filled bars) show an even distribution of lengths, which implies linear growth. Data was obtained using IFA for the flagellar protein PFR and individually measuring the flagella of one hundred cells. Old flagella (open bars) were also measured and are shown to the left of the new flagellum for each cell, however, no relationship between the length of the old PFR and the length of the new PFR is apparent. An approximate value of 8 μm/hour for rate of new flagellum outgrowth was determined from a known (6.3 hr) cell cycle length and an approximate percentage (54%) of the population possessing two flagella.

Five Phases of Slender Cell Division

The apparent linearity of new flagellar outgrowth allowed for its use as a marker in establishing the temporal order of events for kinetoplast and nuclear segregation. Flagella were stained by IFA for PFR and cells were counterstained with the DNA chelating fluor DAPI to visualize the nucleus and kinetoplast. One hundred trypanosomes with two flagella were measured for new flagellum length. They were then categorized into phases that could be easily discriminated by DAPI staining pattern and which could be used usefully as temporal reference points in dissecting the events in the latter part of the bloodstream form trypanosome cell cycle. These phases were then placed into a temporal order according to their mean flagellar length (Fig. 2). Phase 1 trypanosomes have a single nucleus and either one dumbbell shaped kinetoplast, (as shown in Fig. 2a, panel 1), or two kinetoplasts less than 1.5 μm apart. Phase 2 encompasses trypanosomes which are generally premitotic but in which the kinetoplasts are clearly distinct and positioned between 1.5 μm and 2.5 μm apart (Fig. 2a, panel 2). Phase 3 encompasses mitosis and was defined as is those forms that have kinetoplasts over 2.5 μm but which have not yet fully segregated their nuclei, (Fig. 2a, panel 3). Phase 4 consists of post-mitotic forms with two kinetoplasts separated by more than 2.5 μm and with two discrete nuclei. Phase 5 forms were relatively rare (6 from 100 cells). These near-cytokinesis forms had two nuclei and two kinetoplasts separated by less than 2.5 μm.

This study establishes the order in which bloodstream form trypanosomes segregate their nuclear and mitochondrial genomes. It determines that kinetoplast segregation occurs prior to nuclear segregation, as is the case with procyclic forms (Sherwin and Gull 1989). However, it also establishes that kinetoplasts never occupy an anterior nuclear position in bloodstream “dividing-forms” as they do in procyclic “dividing-forms“ (Sherwin and Gull 1989). Importantly, none of the dividing forms possessed only one kinetoplast and two nuclei. From this study, we can begin to build a picture of the co-ordination of organellar segregation and repositioning with outgrowth of the new flagellum prior to cytokinesis. These observations are consistent with classical descriptions of bloodstream cell types (Bruce et al. 1914; Hoare 1956; Wenyon 1926) and of trypanosome cell division in other salivarian species (Stephen 1962). From this study it was possible to categorize “dividing-forms”, using only DAPI staining, into readily recognizable, sequential stages. This is useful because it allows protein expression patterns discerned by IFA to be correlated with cell cycle status.

Mitochondrial Segregation During Slender Cell Division

Having established the temporal order of kinetoplast and nuclear segregation we turned our attention to the manner in which the slender form mitochondrion is segregated. In order to do so we stained trypanosomes for trypanosome alternative oxidase using IFA and counterstained with DAPI. Over two hundred dividing forms were observed and photographed and DAPI staining was then used to group

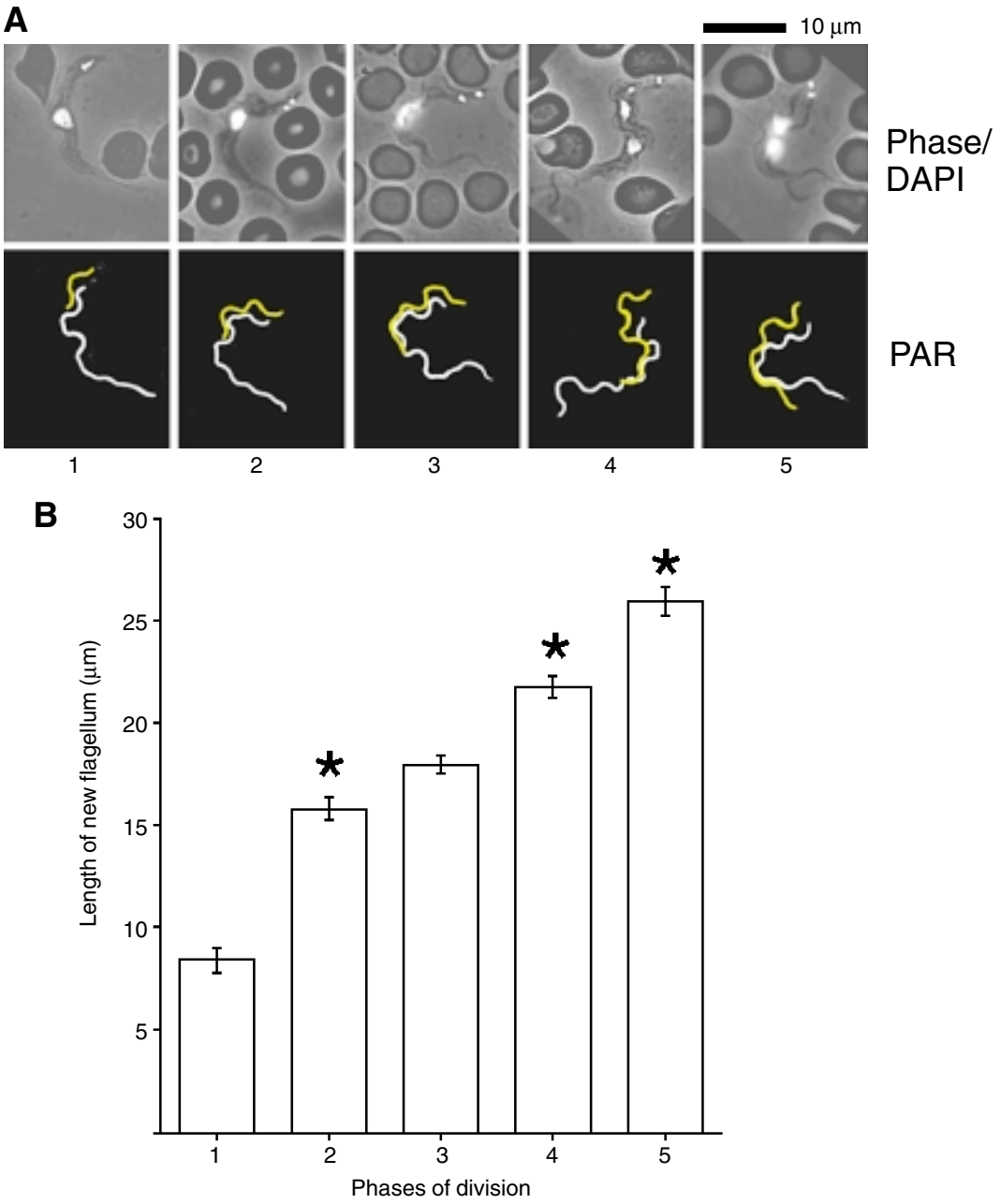

Figure 2. Outgrowth of the new PFR is a temporal marker of the bloodstream form cell cycle. Interkinetoplast distance (id) and number of nuclei (N) were used to define 5 stages of division in 100 cells with two flagellum: 1) 1N id < 1.5 µm [52/100 cells]; 2) 1N id = 1.5–2.5 µm [11/100 cells]; 3) 1N (often mitotic), id > 2.5 µm [15/100 cells]; 4) 2N id > 2.5 µm [16/100 cells]; 5) 2N id < 2.5 µm [6/100 cells]. Fig. 2A shows phase contrast images with DAPI fluorescence of the nuclei and kinetoplasts at each phase (above) with the corresponding image (below) showing IFA of the PFR, which was used as a marker of flagellar length. Flagella were visualized by IFA for PFR. The new flagellum (yellow) was pseudocoloured with adobe photoshop to aid discrimination from the old flagellum (white). Fig. 2B shows the mean new flagellum length at each stage of division. Error bars show one standard error about the mean. The significance of the differences between the means was also tested by a one way analysis of variance * indicates a significant increase in mean new flagellum length from the previous phase.

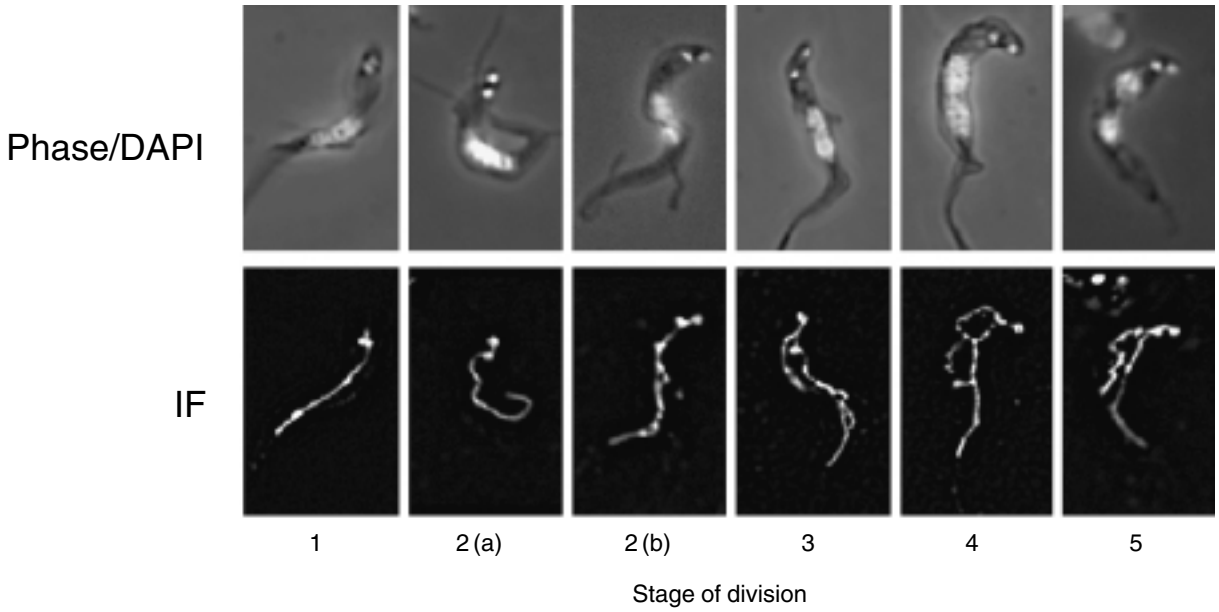

Figure 3. Mitochondrial segregation. The figure shows phase-contrast /DAPI images (upper panel) of six dividing-forms. Forms shown are representative of each of the five temporal stages defined in Fig. 2. Initially only a single tubule is visible although the kinetoplast regions can be seen to segregate (1 and 2a), just prior to mitosis, and still during phase 2 of division, two "loops" arise from the mitochondrion (2b). These loops increase in size (3, 4) until a new mitochondrion is formed by fusion of the lateral processes from each of the loops and by fission of the connections between the nascent mitochondria from the original anterior loop (5).

cells into the five stages of cell division described above (Fig. 3).

In phase 1, a point of bright staining is present which colocalizes with DAPI staining of the kinetoplast together with a thread of staining which runs through the length of the cell. In phase 2, the point of bright has divided and has separated along the single mitochondrial tube as the kinetoplasts segregate. Branching of the chondriome (the mitochondrial tubule) occurs during mitosis or phase 3, with segregation appearing to proceed initially by the formation of two loops, after which these loops expand and the posterior loop fuses to the posterior kinetoplast. In phase 4, following mitosis, the posterior loop fuses to the anterior loop. Finally, in phase 5, the newly formed mitochondrion is detached from the original mitochondrion.

Flagellar Length is a Marker of Differentiation to Stumpy Forms

Early studies have established free flagellar length (Bruce et al. 1914) and cell length (Hoare 1956) as morphological markers for discriminating between slender and stumpy forms. We wondered if a major component of both these parameters – the flagellar length itself would serve as a marker of differentiation. We have previously shown DHLADH to be a definitive marker for intermediate and stumpy forms. When expressed DHLADH localizes to the mitochondrion, which runs the length of the trypanosome on the opposing side to the flagellum and undulating membrane (Tyler et al. 1997). We therefore compared the mean flagellar length of 100 slender forms (lacking DHLADH expression) with the mean flagellar length of 100 intermediate and stumpy forms (expressing DHLADH) (Fig. 4.). All 200 cells had one nucleus and one kinetoplast and all were photographed and measured from the same slide, a blood-smear taken made from a murine infection. We found an unequivocal correlation between flagellar length and differentiation ($p < 0.001$ using Student's t-test), the mean flagellar lengths of the slender form (27 μm) being nearly a third longer than the mean length of the intermediate and stumpy forms (19 μm).

New Flagellum Length can be Used as a Marker of the Differentiation-Division

We have shown that the commitment to differentiation is made prior to a final differentiation division (Tyler et al. 1997). Moreover, that the flagellar length of intermediate and stumpy forms is generally shorter

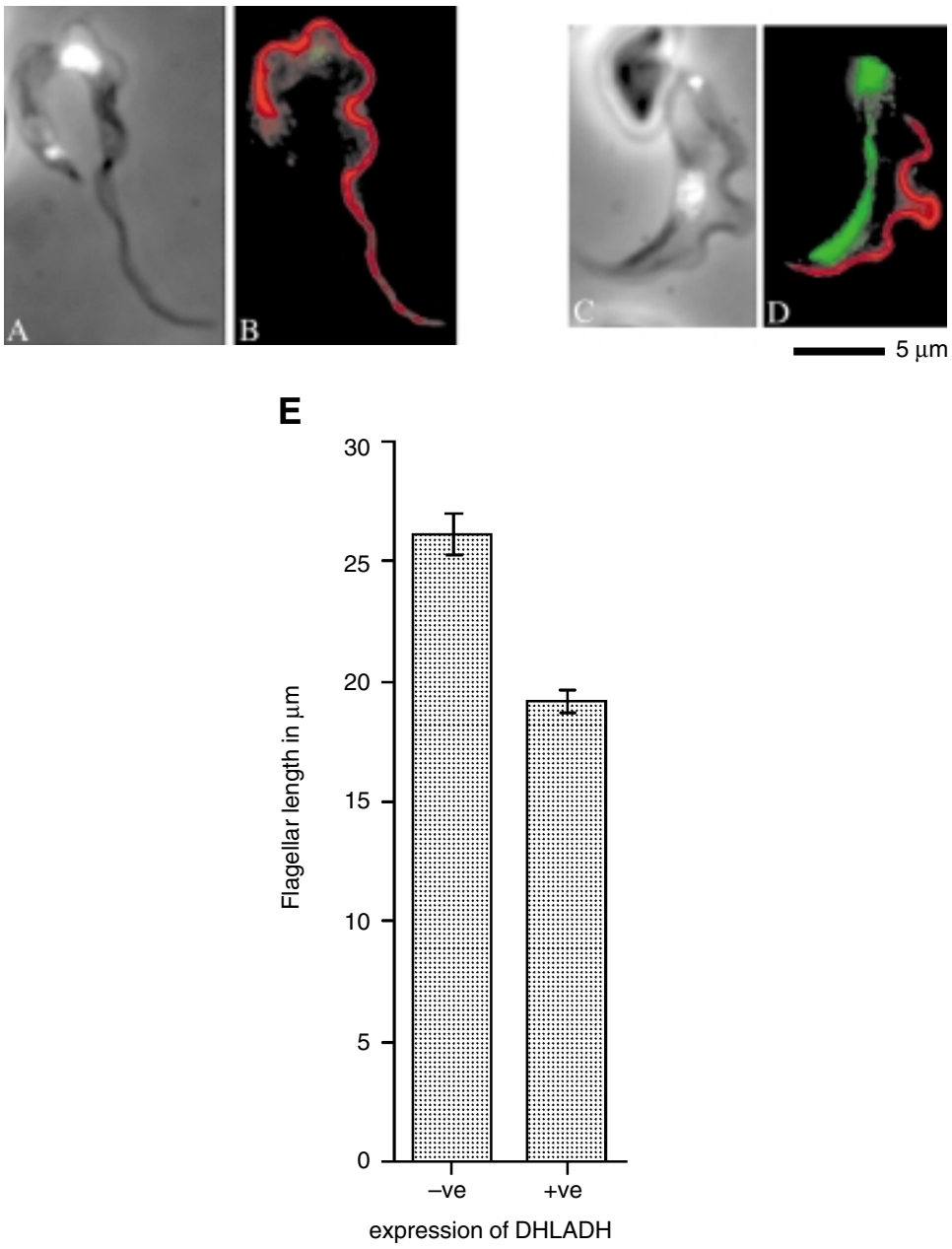

Figure 4. Mean flagellar length shortens during differentiation to the stumpy form which is marked by acquisition of DHLADH. Double IFA was conducted on a mixed population of trypanosomes. PFR expression was used as a marker of flagellar length. Acquisition of DHLADH expression was used to assess cellular differentiation. Merged phase-contrast and DAPI fluorescence image of a slender form (A) and a stumpy form (C). Merged double immunofluorescence, DHLADH expression (FITC, green), PFR expression (TRITC, red). Longer flagella are seen when no DHLADH is expressed (B) and shortened flagellla when DHLADH is expressed (D). E compares the mean flagellar lengths of DHLADH expressing and non-expressing forms. Error bars represent one standard error about the mean.

than that of slender forms, or that reached by the new flagellum during normal slender division (Fig. 4). Bearing these results in mind, we considered that a cell committed to both differentiation and division might make a shorter new flagellum rather than making a flagellum of the usual length that would have to shorten following cytokinesis. Consequently, we searched for evidence of reduced new flagellar outgrowth in cells undergoing a differentiation-division. We searched for phase 5 cells – near-cytokinesis

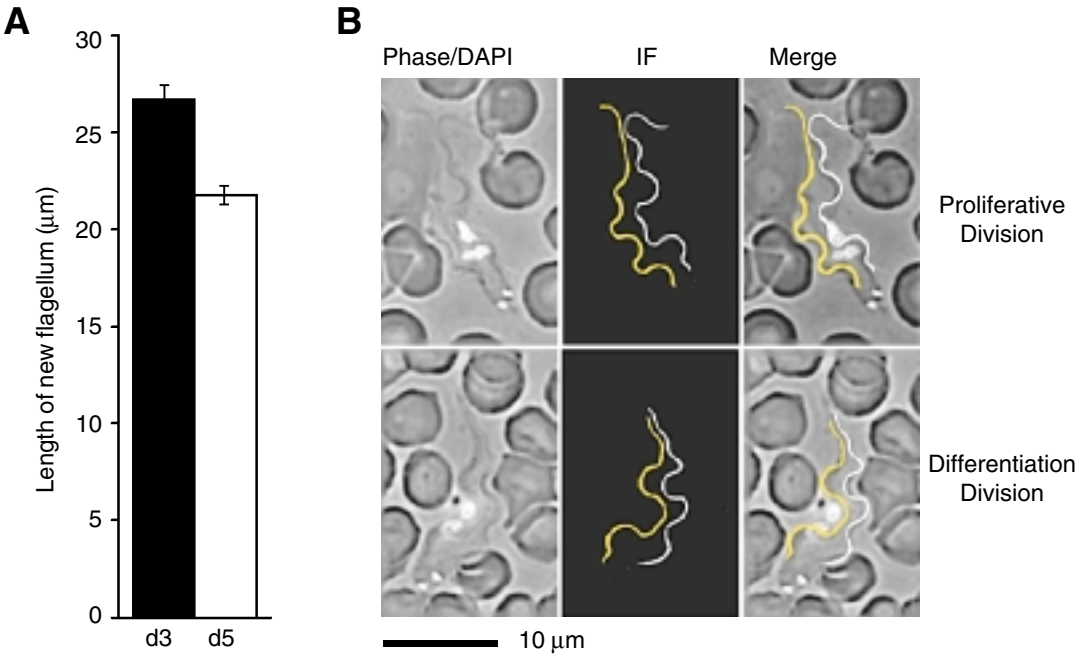

Figure 5. New PFR length can be used to identify cells undergoing the differentiation-division. In Fig. 5A The lengths of the new flagellum for phase 5 forms were compared between a proliferating (day 3 postinfection, filled bar) population and a differentiating (day 5 postinfection, open bar) population and were found to be significantly different. In Fig. 5B, following staining of the PFR by immunofluorescence, dividing-forms were photographed and the length of the new flagellum measured. The new flagellum is shown in yellow and the old PFR in white for convenient identification. Near-cytokinesis forms, which had new flagellum lengths more than 25 μm, were proposed to be in a proliferative division (upper panel). Those that had a new flagellum length of less than 21 μm were proposed to be in a differentiation-division (lower panel). The Figure shows a direct comparison of representative cells of each type in order to highlight the differences in morphology. In particular the appearance of the nuclei is considerably different. The nuclei are stained with DAPI and merged with the phase contrast image in the left-hand panels.

forms with two nuclei and two kinetoplasts separated by less than 2.5 μm – in predominantly slender (day 3 post-infection) and predominantly intermediate and stumpy (day 5 post-infection) populations. We have previously shown that the latter populations contain a higher proportion of cells undergoing a differentiation-division (Tyler et al. 1997). Since we found during this study that phase 5 forms represented only 6% of the early population and 16% of the latter population it is possible that on average phase 5 takes longer in the latter population.

We compared the mean new flagellar lengths between these two populations (Fig. 5a) and found that phase 5 cells from the latter population have significantly shorter new flagella. This suggested to us that it should be possible to discern cells undergoing a differentiation division on the basis of new flagellum length. We compared such cells directly and two representative cells are shown (Fig. 5b). In addition to the difference in length of the new flagellum, the two forms differed morphologically – particularly with respect to the nuclear shape. In the case of phase 5 forms apparently undergoing proliferative division, the nuclei appeared oval, as slender form nuclei do. In contrast, the phase 5 cells apparently undergoing a differentiation-division showed the characteristic roundness of stumpy and intermediate form nuclei and in such cells the nucleoli were generally more pronounced. Trypanosomes in their differentiation-division also appeared to be predominantly broader, and shorter, than the proliferative division forms. In Figure 5b the putative “differentiation-division” cell appears to have both a shorter old flagellum and a shorter new flagellum than the “proliferative-division” cell. In looking at many such cells, however, the result for the old flagellum was not consistent. Although, the mean old flagellum length of phase 5 “differentiation-division” cells was shorter (mean = 26.7 μm, s.e. = 1.6 μm) than phase 5 “proliferative-division” cells (mean = 27.3 μm s.e. = 2.5 μm), a statistically significant difference from the two groups assessed could not be derived.

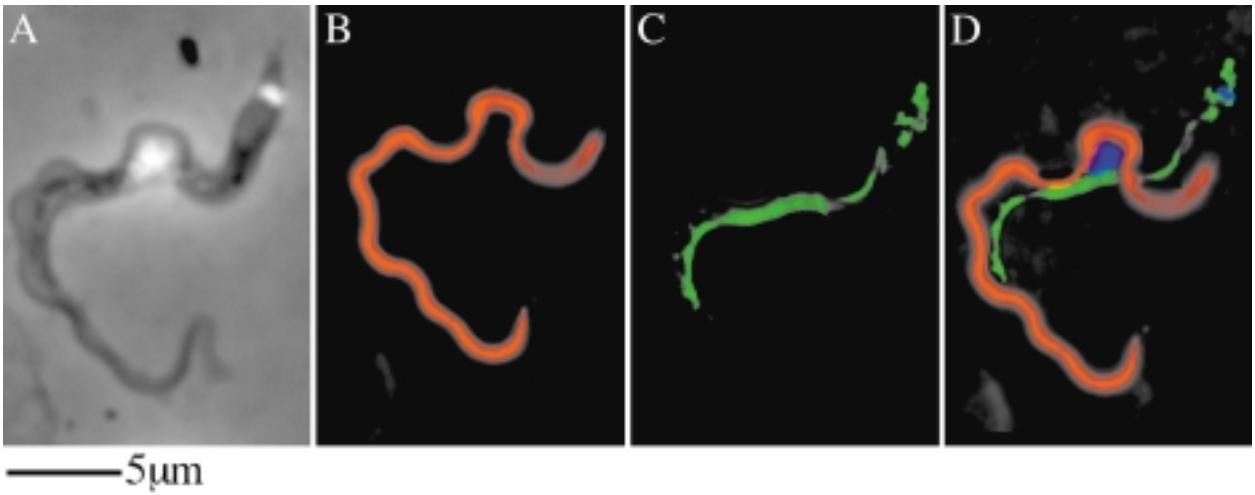

Figure 6. A "long intermediate" form trypanosome. A is the phase contrast DAPI image of the trypanosome which typically has a slender morphology, B, (TRITC) is stained with α-PFR antibody which marks the flagellum. The PFR of this example is over 28 μm long. C, (FITC) Staining for DHLADH expression in the mitochondrion. D shows the merged fluorescence images, allowing the mitochondrion (green) to be clearly discriminated from the flagellum (red). Also included is the localization of the nucleus and kinetoplast by DAPI fluorescence (blue).

Cells Inheriting the Old Flagellum also Differentiate

We have previously proposed that the slender to stumpy differentiation program incorporates a round of cell division (Tyler et al. 1997). It was not clear, however, whether both of the progeny of this division are able to differentiate or whether one remains a slender form and one becomes stumpy. The data above suggested that the cell inheriting the new flagellum becomes a stumpy form. We reasoned, therefore, that if the fate of the cell inheriting the old flagellum was to be a slender form, then one would never see intermediate or stumpy forms with flagella greater than 22 μm in length.

We chose to look for such cells in a population of trypanosomes in which 45% of cells expressed DHLADH by IFA (4 days post-infection). We were able to find that over 10% of cells at this time point (11 from 100 measured) expressed DHLADH and were greater than 22 μm and some, like the one shown (Fig. 6), were close to 27 μm in length. These data suggest a differentiation-division which produces two unequal siblings both of which go onto become stumpy forms. If this is the case than we would surmise that the cell inheriting the old flagellum must activate a program of flagellar shortening which would not be required in the case of the cell inheriting the new flagellum.

Discussion

In eukaryotes, a tightly controlled program of cell division is required to ensure replication and segregation of all essential components of the cell once per cell cycle. In trypanosomes, aspects of these processes are particularly apparent, due to the number of prominent single copy structures (Sherwin et al. 1987; Woodward et al. 1989). Cell division can be further complicated when it must accommodate additional morphological and biochemical changes as part of a cellular differentiation pathway (Zhang 1999). In order to accomplish this successfully, cytoskeletal remodelling and organellar positioning and development must be tightly controlled, since minor variations in any of these processes can clearly be lethal to the organism.

Slender trypanosomes proliferate free in the bloodstream of their mammalian host. The order in which structures are segregated by slender forms has not been previously studied but numerous studies assumed analogy with the segregation profile of procyclic forms (e.g. Chaves et al. 1998; Mutomba and Wang 1996; Tyler et al. 1997; Vassella and Boshart 1996). In general, this study serves to validate this analogy and builds on classical morphometric studies on the bloodstream forms of related trypanosomes (Stephen 1962; Wenyon 1926). We first demonstrated that outgrowth of the new flagellum in slender

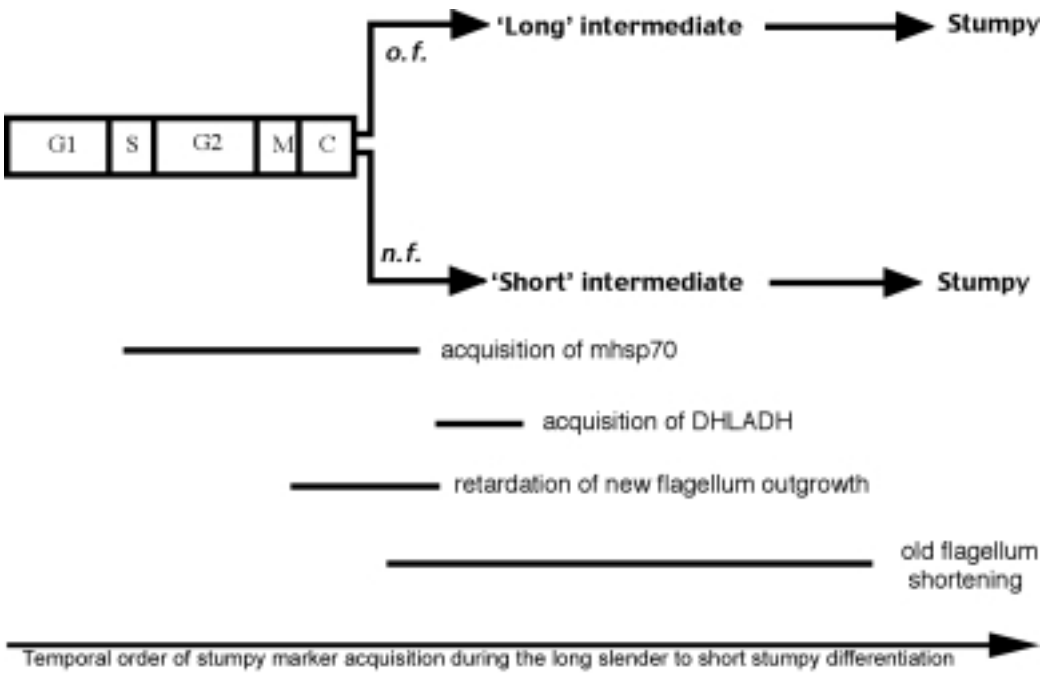

Figure 7. A working hypothesis for the temporal events of the slender-to-stumpy differentiation. It is proposed that the slender-to-stumpy differentiation incorporates an obligatory differentiation-division and that both daughter cells go on to become stumpy without further division. This division is asymmetric in so far as the daughter cell inheriting the old flagellum ***(o.f.)*** can be considerably longer than the one inheriting the new flagellum ***(n.f.)***. This necessitates a program of flagellar shortening which the daughter inheriting the new flagellum may not require. A relative map of the differentiation events examined is shown. Acquisition of MHSP70 and retardation of the new flagellum is detectable during cell division. Acquisition of DHLADH and old flagellar shortening occurs predominantly thereafter.

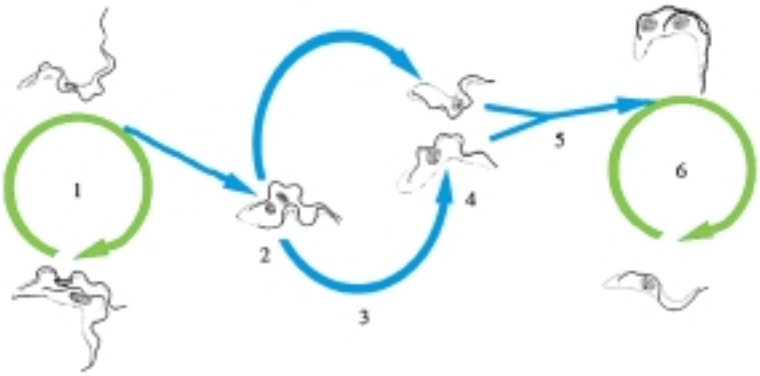

Figure 8. Overview of the proposed pathway of differentiation from a slender via stumpy to the procyclic form. 1) The slender form can continue to undergo rounds of proliferative cell division. 2) The slender form can become committed to differentiation to the stumpy form. When this occurs, the slender form undergoes a heterogeneous cell division, the differentiation-division. At this stage early mitochondrial markers such as mhsp70 are recruited to the mitochondrion. The length reached by the new flagellum prior to cytokinesis is shorter than in previous rounds of proliferative division, and the nuclei formed after mitosis appear more rounded. 3) The daughter cells formed from a differentiation-division then differentiate via a range of morphological intermediates. Most mitochondrial markers, including DHLADH, are recruited during this period and flagellar shortening occurs. 4) Both of the daughter cells of the differentiation-division become stumpy forms. 5) Under appropriate conditions, or when taken up by a tsetse-fly, these stumpy forms will acquire procyclin, shed VSG, complete mitochondrial biogenesis, undergo morphogenesis and re-enter the proliferative cell cycle.

forms occurs at an approximately linear rate obtaining a crude estimate of 8 μm per hour which is twofold higher than in procyclic forms (3.6 μm per hour) (Bastin et al. 1999). The linear rate of flagellar outgrowth allowed its use as a temporal marker of the cell cycle, to demonstrate that the segregation of the mitochondrial genome of the slender form does precede segregation of the nuclear genome as previously observed in the procyclic form (Sherwin et al. 1987).

Interestingly, segregation of the mitochondrial tubules themselves only begins after segregation of the kinetoplast and it is not completed until just prior to cytokinesis. While it is well established that segregation of the mitochondrial genome is microtubule mediated, since the kinetoplast itself is hard-wired to the basal body in these organisms (Robinson et al. 1991). It remains an open question as to how the position of the slender mitochondrion is maintained and how it is triggered to branch and fuse in a cell cycle dependent manner. The mechanism of segregation appears to be analogous to that described for leishmainia (Simpson and Kretzner 1997), in so far as at one stage a branched loop is formed which is effectively cleaved into two new linear tubules. The close proximity of the trypanosome mitochondrion to the subpellicular array (Brown et al. 1973) is comparable to the positioning of the mitochondrion with respect to the microtubule described in fission yeast (Yaffe et al. 1996). It may be that there is an association between the mitochondrion and the cytoskeleton, an association which is supported by findings in *Drosophila* in which mitochondrial positioning is regulated in part by a close association with the cytoskeleton using microtubule motors (Periera et al. 1997).

In response to a density dependent, apparently cAMP mediated signal, trypanosomes begin to differentiate to the non-proliferative, stumpy form (Vassella et al. 1997). In earlier studies we showed that the onset of differentiation occurs prior to a final cell division that differs from previous divisions. We showed using classical morphological criteria that the cells emergent from this division appeared slender, but that at least one of the cells went on to become stumpy via the spectrum of morphological intermediate forms (Tyler et al. 1997).

In this study we showed that the onset of morphological change can be detected during the differentiation-division. In particular, the new flagellum does not appear to reach the same length that it does during a proliferative division. This is an indication of precommitment by the daughter cell inheriting the new flagellum to becoming a stumpy form. If only one cell was committed to becoming stumpy then the flagellar length of intermediate forms should not exceed the length of the new flagellum during the differentiation-division. However, many intermediate forms were evident that were considerably longer than this. Our interpretation, therefore, is that both cells arising from the differentiation-division go on to become stumpy forms. In the case of the cell that inherits the old flagellum, however, a program of flagellar shortening must be initiated, either shortly before or shortly after cytokinesis of the differentiation-division. Our working hypothesis for the temporal pathway of the slender to stumpy differentiation is sketched below (Fig. 7). It is also possible to observe differences in the cell length and nuclear structure of cells close to cytokinesis, which are likely to be indicative of commitment to the production of a stumpy form. These nuances of form are likely to be able to facilitate future studies on the slender to stumpy differentiation pathway.

It should be noted, however, that we were unable to show cytologically that the differentiation-division observed here is obligatory and the sole pathway of slender to stumpy differentiation and it may be that some G_1 slender forms differentiate directly to stumpy forms without undergoing an intervening cell division. Indeed the rapid kinetics observed in vitro by others (Vassella et al. 1997) and our own computer modelling of the parasitaemic time course (Tyler et al. 2001) tend to favour this explanation. Finally, in light of the data in this paper and using figures sketched by Hoare (1956), we have temporally ordered commonly observed trypanosome morphologies along a pathway of differentiation from slender to the procyclic forms (Fig. 8).

Methods

Trypanosomes: For all bloodstream parasitaemias a pleomorphic line of *T.b.rhodesiense* EATRO 2340 (McLintock et al., 1990) was used as previously described (Tyler et al. 1997). 1×10^6 trypanosomes were inoculated into immunocompetent BALB/c mice. Typically >90% slender form populations were obtained on day 3 postinfection at parasitaemias of less than 5×10^7/ml. Typical populations obtained on day 5 postinfection were > 90% stumpy by morphology at parasitaemias of $>1\times10^8$/ml. Bloodstream trypanosomes were purified over a DEAE cellulose column (as described by Lanham 1968).

Immunofluorescence assay (IFA): Purified, PBS washed trypanosomes were prepared for IFA by settling them for 10 minutes on organosilane treated slides, rinsing in PBS and fixing in methanol at –20 °C or in 4% paraformaldehyde. IFA was performed as previously described (Sherwin et al. 1987). Briefly,

samples were rehydrated in PBS for 5 minutes, primary antibodies were applied for 1 hour in a humid chamber at room temperature, samples were washed three times in PBS for 5 minutes per wash, secondary antibodies were then applied and the process was repeated. Finally, 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole dichydrochloride: Boehringer Mannheim, Indianapolis, IN) was applied for 30 seconds and the sample washed in distilled water before mounting. Microscopy was performed using a Leica DMRXA fluorescence microscope via a ×100 oil immersion planar fluorotar objective lens. Resultant images were captured using cooled charge coupled device (CCD) (Photometrics series 200: Munich, F.R.G.) with IPlab spectrum software. The measure feature of IP lab facilitated the measurement of flagellar length from the captured image. The images presented were processed using Adobe Photoshop 3.0. To provide a quantitative indication of flagellum lengths we measured the lengths of the paraflagellar rods, as stained by IFA with the L13D6 anti-PFR A and B monoclonal antibody (Kohl et al. 1999). The paraflagellar rod (PFR) is present for the full length of the flagellum outwith of the flagellar pocket and so measurement of its outgrowth during the trypanosome cell cycle closely corresponds with flagellar dimensions obtained by electron microscopy (Sherwin and Gull 1989). Two other primary antibodies were used for visualization of the trypanosome mitochondrion as previously described (Tyler et al. 1997) – a murine monoclonal antibody to trypanosome alternative oxidase (TAO) and a rabbit polyclonal antibody to trypanosome dihydrolipoamide dehydrogenase (DHLADH).

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