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Cytokinesis in *Trypanosoma brucei* differs between bloodstream and tsetse trypomastigote forms: implications for microtubule-based morphogenesis and mutant analysis

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Summary

Trypanosomes use a microtubule-focused mechanism for cell morphogenesis and cytokinesis. We used scanning electron and video microscopy of living cells to provide the first detailed description of cell morphogenesis and cytokinesis in the early-branching eukaryote *Trypanosoma brucei*. We outline four distinct stages of cytokinesis and show that an asymmetric division fold bisects the two daughter cells, with a cytoplasmic bridge-like structure connecting the two daughters immediately prior to abscission. Using detection of tyrosinated α-tubulin as a marker for new or growing microtubules and expression of XMAP215, a plus end binding protein, as a marker for microtubule plus ends we demonstrate spatial asymmetry in the underlying microtubule cytoskeleton throughout the cell division cycle. This leads to inheritance of different microtubule cytoskeletal patterns and demonstrates the major role of microtubules in achieving cytokinesis. RNA interference techniques have led to a large set of mutants, often with variations in phenotype between procyclic and bloodstream life cycle forms. Here, we show morphogenetic differences between these two life cycle forms of this parasite during new flagellum growth and cytokinesis. These discoveries are important tools to explain differences between bloodstream and procyclic form RNAi phenotypes involving organelle mis-positioning during cell division and cytokinesis defects.

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Introduction

Protists are usually immediately recognizable by their cell shape. The replication of this shape through a cell cycle involves a series of cellular morphogenetic events and then a cytokinesis event. Two types of cell cycle are often recognized – the proliferative type, which leads to two daughter cells with the same characteristics as the original, and the differentiation type, which leads to the formation of at least one daughter cell which differs from its parent. The former type of proliferative cell cycle was historically referred to as 'binary fission'. However, it is now clear that the cellular morphogenetic process leading to the formation of two seemingly identical daughters often involves asymmetry in the formation and positioning of structures within the dividing cells and resulting daughters. Examples of this can be found in the cell division cycles of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (for reviews see Pollard and Wu, 2010; Oh and Bi, 2011). For most other protists although there are excellent descriptions of cell shape, form and cytoplasmic organization little is known on how these are reproduced throughout the cell cycle and the nature and inheritance patterns of the two daughter cells. In this study we provide detailed analysis of such events in two important proliferative life cycle stages of the African trypanosome *Trypanosoma brucei*, a notable pathogen and an example of a protist which achieves cytokinesis with a cytoskeleton almost entirely composed of microtubules.

Underlying the *T. brucei* plasma membrane is the subpellicular microtubule array, which defines the characteristic shape of trypanosomes (Vickerman, 1962; Anderson and Ellis, 1965; Sherwin and Gull, 1989b). This microtubule cytoskeleton remains intact during all stages of the cell cycle; thus the two daughter cells are produced within the confines of this array and the array must undergo significant spatial and temporal re-modelling (Sherwin and Gull, 1989b). Animal cells use the actinomyosin contractile ring to drive in-folding of the plasma membrane (called furrow ingression) to delineate two daughters, and genomic analysis indicates it is likely the genes for this process were present in the common ancestor of all eukaryotes. However, the bikonts (which include trypanosomatids) lack myosin-II (Richards and Cavalier-Smith,

2005; Baluska *et al*., 2006; Foth *et al*., 2006) and RNAi knockdown of actin in *T. brucei* procyclic (tsetse) form trypomastigotes indicates that it is not essential for division (García-Salcedo *et al*., 2004). The mechanism by which cytokinesis is achieved must, therefore, differ significantly from the well-analysed metazoa (Farr and Gull, 2012). Actinomyosin-independent cytokinesis is observed in plants which also lacks myosin II, where there is no in-folding of membrane; instead membrane fusion events at the microtubule-rich phragmoplast achieve formation of two separate plasma membranes (Müller *et al*., 2009). Addressing how cytokinesis is achieved in a bikont protist and whether it is similar to actinomyosin-independent cytokinesis of plants, is therefore of general biochemical interest in understanding how cytokinesis is achieved in more diverse organisms.

Trypanosoma brucei undergoes a complex life cycle; alternating between proliferating and non-proliferating life cycle forms during transit between the tsetse fly vector and mammalian host (for review see Steverding, 2008). Proliferative and differentiation division events must occur throughout its life cycle to guarantee survival, adaptation and transmission. Following the discovery of *T. brucei* as the causative agent of sleeping sickness and nagana in sub-Saharan Africa, techniques for axenic culture of two of the proliferating life cycle stages, the procyclic form (found in the midgut of the tsetse fly) and the mammalian bloodstream form, were developed and are widely used in cell and molecular studies of *T. brucei* biology, life cycle stage differentiation, pathogenicity, and as a model organism for analysis of the flagellum and cytoskeleton. In our previous work, we established descriptions of cell cycle, cytokinesis and cellular morphogenesis in the procyclic form of *T. brucei* (Sherwin and Gull, 1989a; 1989b; Robinson *et al*., 1995). We demonstrated that the sub-pellicular array is not disassembled during division and that new microtubules are added to the existing cortex as the cell cycle progresses, with both daughter cells inheriting a mixture of old and newly assembled microtubules (Sherwin *et al*., 1987; Sherwin and Gull, 1989b; Woodward and Gull, 1990). Using pharmacological insults, we revealed that trypanosomes possessed unusual 'dependency' relationships between cellular structures and events during the cell cycle – a fact that has been confirmed and extended by others (Ploubidou *et al*., 1999; Tu and Wang, 2004; Hammarton *et al*., 2007). Morphogenesis in the cell cycle therefore involves both semi-conservative (sub-pellicular array) and conservative (flagellum, flagellum attachment zone, basal body) inheritance patterns (for review see Gull, 1999). However, how this division process compares with the bloodstream form life cycle stage, where there are many known organelle and biochemical differences (Vickerman, 1962; 1969; Anderson and Ellis, 1965), and precisely how the microtubule cytoskeleton achieves cytokinesis in either life cycle stage are not known.

The application of RNAi technologies in *T. brucei* has led to a rapidly increasing number of mutant phenotypes being described, often including failure in cell division. Given the growing importance of these post genomic studies we were struck by the fact that there has been no concerted attempt at a careful analysis of cytokinesis in trypanosomes or a discrete comparison of the morphogenesis process and cytokinesis between the two most studied proliferative forms – bloodstream and procyclic form trypomastigotes. Analyses of RNAi knockdowns of the same protein often reveal phenotype differences between the two major life cycle forms (e.g. Hammarton *et al*., 2003; Tu and Wang, 2004; Kumar and Wang, 2005; Broadhead *et al*., 2006; Rothberg *et al*., 2006), suggesting some differences. Studies vary tremendously in the depth of analysis but nevertheless there is a general theme of variation between the two major trypomastigote forms and in the types of cytokinetic mutant phenotypes produced. The lack of a comparative analysis of overall cellular morphogenesis during the cell cycle and the cytokinesis process is a serious hindrance to meaningful interpretation of such mutant phenotypes.

Here we provide a comparative analysis of division and cytokinesis analysis using fluorescence markers, electron and live cell microscopy outlining the major morphogenetic stages of the cell cycle in both procyclic and bloodstream forms to identify the shared and distinct mechanisms used by both life cycle stages to achieve cytokinesis. We show that an asymmetric division fold bisects the two daughter cells, the placement of which differs in the two life cycle stages, and a discrete cytoplasmic bridge connects the two daughter cells immediately prior to abscission. We have dissected the underlying spatial and temporal re-modelling of the microtubule cytoskeleton using markers for new or growing microtubules and location of a plus end binding protein XMAP215. These demonstrate spatial asymmetry in the underlying microtubule cytoskeleton throughout the cell division cycle and identify the major regions of growth and reorganization. These discoveries and differences between the two life cycle forms have implications for both understanding cytokinesis in an organism with a microtubule dominated cytoskeleton and past and future studies on the differing phenotypes in cell morphogenesis in the procyclic and bloodstream form stages of this parasite.

Results

Procyclic and bloodstream forms have related but distinct morphogenetic events leading to cytokinesis

To compare the morphological changes during division of these two life cycle stages we performed a comprehen-

sive analysis by SEM. In order to identify and characterize minor stages in the cell cycle many thousands of images were examined for each cell form and categorized by at least two independent observers.

Cell division in the procyclic form

Procyclic trypanosomes have a long slender form with a single flagellum that exits the flagellar pocket at the posterior end of the cell and is attached along the length of the cell body (Fig. 1A). The first external indication of cell cycle progression is a new flagellum that exits the flagellar pocket and whose tip is connected to the old flagellum (arrow) via a transmembrane mobile junction – the flagella connector (FC) (Fig. 1B) (Moreira-Leite *et al.*, 2001; Briggs *et al.*, 2004; Davidge *et al.*, 2006). This connection is maintained as the new flagellum grows in an anterior direction and the two flagellar pockets segregate (Fig. 1C). The exit point from the flagellar pocket of the new flagellum (Fig. 1C; arrow) is always positioned posterior to that of the old flagellum (arrowhead) along the long axis of the cell. The new flagellum is always positioned to the left of the old when viewed from cell's posterior end (Sherwin and Gull, 1989b) (Fig. 1C). During this time the flagella begin to segregate, and are widely separated around the circumference of the cell near the posterior but more closely adjacent towards the anterior (Fig. 1C). Here we define four characteristic stages of cytokinesis: division fold generation, division furrow ingression, pre-abscission and abscission. In procyclics the division fold is an invagination of the cell body between the two flagella, which facilitates the early definition of two nascent daughter cells (Fig. 1D; arrow). The fold does not extend all the way to the posterior end of the dividing cell, but stops some way back from this (Fig. 1D and E; circled). This defines two nascent posterior ends; a nascent posterior end associated the daughter cell with the new flagellum (called the new-flagellum daughter) (Fig. 1E) and a nascent posterior end associated with the daughter cell with the old flagellum (called the old-flagellum daughter) (Fig. 1E). Even though the fold is placed asymmetrically along only a portion of the original cell, it does bisect the cell approximately by volume (Fig. 1F and G) yielding daughter cells of similar but not identical shape (see later). The second stage in cytokinesis is division furrow ingression characterized by the appearance of a gap between the two daughters (Fig. 1E; arrow). The distal tip of the new flagellum remains attached to the old via the flagella connector during this process (Briggs *et al.*, 2004) (Fig. 1E and F; dashed circle) and the new flagellum has not grown to reach the anterior of the existing cell body (Davidge *et al.*, 2006). In the third stage, pre-abscission, the two nascent daughter cells remain connected via a thin section of membrane linking the posterior end of the old-flagellum daughter cell (Fig. 1G; circled) with the side of the new-flagellum daughter cell, but the link between the flagella (via the flagella connector) is released (Fig. 1G). We call this connection a 'cytoplasmic bridge' (see later). Abscission then follows to produce two uniflagellated daughters.

Cell division in the bloodstream form

Bloodstream form cells also have a single attached flagellum that exits the flagellar pocket at the posterior end of the cell (Fig. 1I). The primary external morphological difference to the procyclic form is a flagellar pocket positioned closer to the posterior. Again, the first indication of cell division is the emergence of a short new flagellum that extends from a flagellar pocket (Fig. 1J; arrow) positioned posterior to the old flagellum (Fig. 1K). As in the procyclic form the flagella begin to segregate, and are more widely separated near the cell posterior (Fig. 1K). The new flagellum is attached to the cell body, but its distal tip is not connected to the old flagellum as in the procyclic form; instead it lies close alongside the old flagellum at it grows (Fig. 1L; dashed circle) with its distal tip embedded in an indentation of the cell body, called the groove (Hughes *et al.*, 2013). Subsequently, the two flagellar pockets segregate and the flagellar pocket of the new flagellum is positioned posterior to and to the left of the flagellar pocket of the old flagellum when viewed from the cell's posterior. The new flagellum is positioned to the left of the old when viewed from the cell's posterior as in the procyclic form (Fig. 1K and L).

We can define the same four characteristic stages of cytokinesis in the bloodstream form as the procyclic: division fold generation, division furrow ingression, pre-abscission and abscission. Fig. 1M illustrates a cell stage where the division fold is present (arrow), but the distal tip of the long new flagellum has grown unattached from the cell body. This is a cell stage that is not found the procyclic from where the distal tip of the new flagellum remains attached until the pre-abscission stage. It was difficult to view more of the division fold in the bloodstream form due to the more 'twisted' nature of these cells. Two distinct posterior ends (Fig. 1M; circled) are evident, but the fold finishes closer to the pre-existing posterior end in a bloodstream form cell than in a procyclic form cell (compare Fig. 1F with M). This difference makes it more difficult to reliably distinguish the old-flagellum daughter cell from the new-flagellum daughter cell at later time points (Fig. 1N and O). In Fig. 1N the division furrow has progressed towards the posterior end of the cell (arrow). Given that there is no flagella connector in bloodstream forms then this leads to a clearly bifurcated cell shape earlier in division than for the procyclic. Subsequently, a pre-abscission stage exists where nascent daughters are apposed and joined at or close to their posterior ends

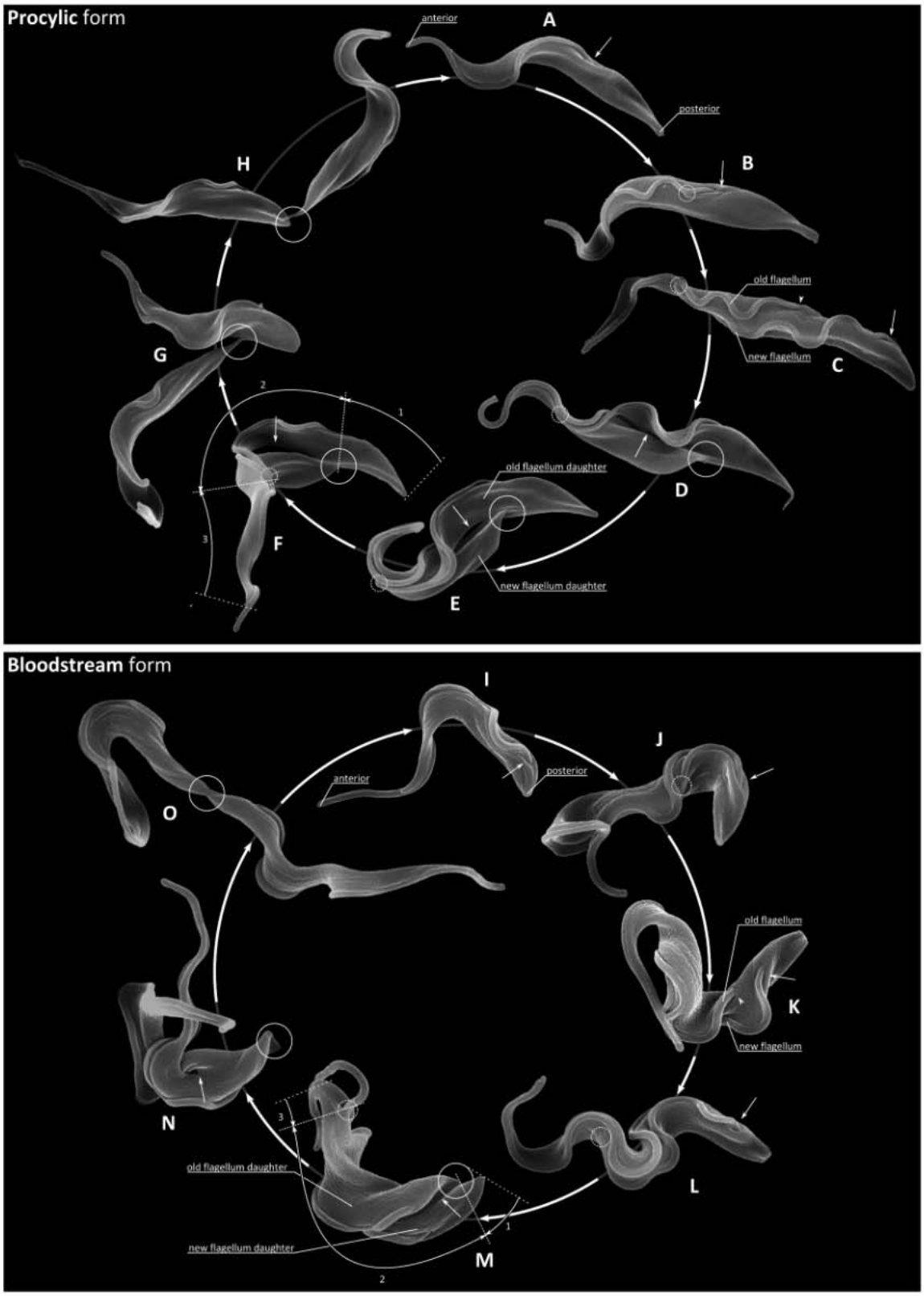
Procylic form
anterior
A
posterior
B
old flagellum
new flagellum
C
D
old flagellum daughter
new flagellum daughter
E
F
G
H
1
2
3
Bloodstream form
I
J
anterior
posterior
old flagellum
K
new flagellum
L
M
N
O
old flagellum daughter
new flagellum daughter
1
2
3

Fig. 1. Differences in external morphology during the cell division cycle of the procyclic and bloodstream forms. Composite scanning electron micrograph images of stages of procyclic (A–H) and bloodstream (I–O) form cell division.
A and I. G1 cell with a single attached flagellum. The exit point of the flagellum from the flagellar pocket is arrowed and the anterior and posterior indicated.
B and J. A new flagellum has grown to extend from the flagellar pocket (arrow). The distal tip of the new flagellum is laterally connected to the old flagellum in the procyclic (B) and is laterally embedded in the groove structure in the flank of the cell in the bloodstream form (J), indicated by dashed circles.
C, K–L. The flagellar pocket associated with the new flagellum (arrowed) is positioned posterior to the flagellar pocket associated with the old flagellum (arrowhead). The new flagellum is located to the left of the old when viewed looking from posterior to anterior.
D and M. A division fold is evident between the two flagella (arrowed) which is located along the long axis and begins to define the daughter cell shape. There are two distinct posterior end profiles. The new-flagellum daughter inherits the existing posterior end and a new posterior end is formed for the old-flagellum daughter (circled). The new flagellum is still attached to the old by the flagella connector in the procyclic form (D), but has grown free of the cell body in the bloodstream form (M), indicated by dashed circles.
E–F, N. A division cleft has opened up between the daughters (arrow) and the new flagellum tip remains attached to the old flagellum by the flagella connector in the procyclic form (E–F).
G–H, O. Pre-abscission stage. In the procyclic form (G–H) the two daughter cells are attached by the posterior end of the old-flagellum daughter (circled) to the side of the new-flagellum daughter by a cytoplasmic bridge connection. In the bloodstream form (O) a posterior-to-posterior (circled) configuration is typical.

(Fig. 1O). The cytoplasmic bridge connection is morphologically different to that typically observed procyclic form (compare Fig. 1H with O) in that it is broader and more substantial, although a similar substantial cytoplasmic bridge was observed on rare occasions in the procyclic. Abscission then follows (usually – see later) to produce two uniflagellated daughters.

Division fold generation is unidirectional and requires precise insertion of microtubules between the two flagella

We found that the first two stages of cytokinesis in procyclic and bloodstream forms were the generation of the lateral fold (Fig. 1D and M) followed by furrow ingression from the anterior to posterior along the line of the fold (Fig. 1F and N), both of which occur along a line between the old and new flagella (Fig. 2A). Although placement of the division fold was different between the life cycle forms, in that the fold finished closer to the pre-existing posterior end in a procyclic form cell, its placement between the two flagella required segregation of the two flagella in both forms. As the sub-pellicular array remains present at all division stages this would be expected to require insertion of microtubules between the old and new flagellum.

We carried out an in-depth analysis of TEM cross-sections of old and new flagella that were either still close to each other (i.e., where no segregation had occurred, as in Fig. 2E) or where segregation of the flagella was underway (Fig. 2F) in both life cycle forms. Prior to segregation the old and new flagella and flagella attachment complexes were closely associated in both forms (Fig. 2E). The four specialized microtubules [called the microtubule quartet (MtQ)] (Taylor and Godfrey, 1969; Lacomble *et al.*, 2009) were associated with each flagellum and were located alongside one another (Fig. 2E; circled). When segregation was underway sub-pellicular microtubules inserted precisely between the MtQ sets (Fig. 2F). A model outlines the insertion of sub-pellicular microtubules between the two flagella for both forms (Fig. 2G–I). This demonstrates that initial invasion must be made between microtubule 4 of the MtQ of the old flagellum and the new flagellum attachment zone (FAZ) filament of the new flagellum (Fig. 2E), representing a unique seam. Further microtubule insertion between old MtQ microtubule 4 and the neighbouring sub-pellicular microtubule highlights a second unique seam where polarity of MtQ number 4 is opposite to that of the neighbouring sub-pellicular microtubule (Fig. 2F).

In cross-sections of dividing cells, invagination of membrane to generate the division fold was always unidirectional in both forms – in-folding occurred between the two flagella, but was never observed in the opposing area of the cell. No specific structural component was observed at the TEM level which could orchestrate indentation of the plasma membrane (Fig. 2B and C). No alteration of the normal spacing between sub-pellicular microtubules was observed in the area of the fold or furrow.

There are distinct areas of microtubule growth and re-modelling during cell division

Our SEM analysis indicated cytokinesis involves an asymmetric fold whose placement differs between procyclic and bloodstream forms. The asymmetric fold placement means that different portions of the existing cell would be inherited by each daughter. We define three zones that undergo distinct cell morphogenetic events and inheritance in both life cycle forms: Zone 1 at the posterior of the mother cell would be inherited by the new-flagellum daughter cell (Fig. 1F and M; Fig. 3A and B; labelled 1). Zone 2 in the middle (Fig. 1F and M; Fig. 3A and B; labelled 2) of the dividing trypanosome is an area of great complexity. It runs from a line close to the emergence of the new flagellum from the flagella pocket to the most anterior point of connection of the new flagellum to the cell body. In this middle