Microtubule Polarity and Dynamics in the Control of Organelle Positioning, Segregation, and Cytokinesis in the Trypanosome Cell Cycle

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Abstract. *Trypanosoma brucei* has a precisely ordered microtubule cytoskeleton whose morphogenesis is central to cell cycle events such as organelle positioning, segregation, mitosis, and cytokinesis. We have defined microtubule polarity and show the + ends of the cortical microtubules to be at the posterior end of the cell. Measurements of organelle positions through the cell cycle reveal a high degree of coordinate movement and a relationship with overall cell extension. Quantitative analysis of the segregation of the replicated mitochondrial genome (the kinetoplast) by the flagellar basal bodies identifies a new G2 cell cycle event marker. The subsequent mitosis then positions one "daughter" nucleus into the gap between the segregated basal bodies/kinetoplasts. The anterior daughter nucleus maintains its position relative to the anterior of the cell, suggesting an effective yet cryptic nuclear positioning mechanism. Inhibition of microtubule dynamics by rhizoxin results in a phenomenon whereby cells, which have segregated their kinetoplasts yet are compromised in mitosis, cleave into a nucleated portion and a flagellated, anucleate, cytoplast. We term these cytoplasts "zoids" and show that they contain the posterior (new) flagellum and associated basal-body/kinetoplast complex. Examination of zoids suggests a role for the flagellum attachment zone (FAZ) in defining the position for the axis of cleavage in trypanosomes. Progression through cytokinesis, (zoid formation) while mitosis is compromised, suggests that the dependency relationships leading to the classical cell cycle check points may be altered in trypanosomes, to take account of the need to segregate two unit genomes (nuclear and mitochondrial) in this cell.

T HE cytoskeleton of eukaryotic cells has been shown to operate in important functions such as cell movement, intracellular transport, division, and the control of cell shape. The basic molecular architecture of the individual components of the cytoskeleton confers particular properties on the final complex high order structure. These properties, such as polarity and polymerization dynamics, provide basic attributes of organelles such as microtubules that are reflected in their modulation during cell cycle shape changes or in the establishment of overall cellular polarity.

The African trypanosome, *Trypanosoma brucei* has a very precisely ordered microtubule cytoskeleton that provides an excellent opportunity to study the relationships between molecular events and acquisition of cellular form. The detergent-extracted cytoskeleton of *T. brucei* retains the overall form of the cell from which it was derived (Sherwin and Gull, 1989a). The cell shape is maintained by a subpellicular corset of microtubules that are cross-linked to each other and to the plasma membrane (Hemphill et al.,

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1991; Souto-Padron et al., 1984). The single flagellum exits the cell body via the flagellum pocket located at the posterior of the cell and is attached to the cell body along its length; its distal tip extends slightly beyond the anterior end of the cell (Sherwin and Gull, 1989a). The mitochondrion of *T. brucei* is unusual among eukaryotes in that it exists in the form of a single elongated tubular structure that extends along the length of the cell. However, the mitochondrial genome is not distributed throughout this single mitochondrion. Rather, it exists as a discrete physical structure, the "kinetoplast." This mass of many thousands of catenated mini-circle DNA molecules and around 25 maxi-circle DNA molecules is located at one end of the single mitochondrion in a distended region adjacent to basal body of the flagellum (Steinert et al., 1976; Ryan et al., 1988; Robinson and Gull, 1991). The highly compact nature of the kinetoplast allows it, along with the nucleus, to be easily visualized with fluorescent cytological probes. Cells early in the cell cycle posses a single kinetoplast and a single nucleus, these cells are termed 1K1N. As the cell cycle progresses the kinetoplast replicates and segregates before the nucleus to produce a 2K1N cell. Cells then enter mitosis, the outcome of which is a 2K2N cell. Finally cytokinesis occurs, cleaving the cell into two 1K1N siblings.

The kinetoplast, basal body, and flagellum are present in

single copies, and their position, replication and segregation are highly regulated within the cell cycle. At least in some cases, this positional ordering and segregation pattern of organelles involves direct interaction with the microtubule cytoskeleton (Robinson and Gull, 1991). In trypanosomes there is a single periodic S phase in the cell cycle for both the nucleus and kinetoplast DNA. We have previously provided a description of the cell cycle timings of periodic events such as nuclear and kinetoplast DNA synthesis phases, basal body duplication, flagellum growth, mitosis, etc. (Woodward and Gull, 1990). These replicative events are coordinated temporally but it also appears that the movements of the duplicated organelles and overall cell shape are spatially ordered with great precision. Even though the classical literature on trypanosomes recognizes that the shape and form of the cell changes both in the life cycle and the cell cycle, we still have little idea of the molecular events influencing such cellular changes.

In this study we have sought to understand the nature of the link between events such as the discrete positioning of organelles and their segregation, mitosis, cytokinesis, and the morphogenesis of the microtubule cytoskeleton. We have been able to define microtubule polarity by three separate assays, we have measured organelle positions in the cytoskeletons throughout the cell cycle and have related these to the kinetics and extent of overall cell extension. Moreover, we have used the antimicrotubule drug rhizoxin (Tsuruo et al., 1986) to inhibit microtubule dynamics during cytoskeletal morphogenesis and so reveal a fascinating phenomenon whereby cells can cleave into a nucleated portion and a flagellated, anucleate cytoplast that contains a kinetoplast and mitochondrion. We term these structures "zoids." Examination of the aberrant drug-affected cytokinesis events provides evidence for the identity of a cytoskeletal domain that acts as a decision site for both the axis and direction of cleavage.

Materials and Methods

Organisms

Procyclic forms of *T. brucei brucei* strain 427 were maintained by subculturing in semi-defined medium (SDM 79) (Brun and Schönenberger, 1979).

Preparation of Whole Mount Cytoskeletons

Preparation of whole mount cytoskeletons was carried out as previously described by Sherwin and Gull (1989a).

Preparation of Sheep Brain Tubulin

Microtubule protein was prepared as described by Roobol et al. (1976). Four Hp$_2$ sheep brain microtubule protein pellets were gently resuspended in 0.5 M PMEG (1 mM MgCl$_2$, 1 mM EDTA, 1 mM GTP, 0.5 M Pipes, pH 6.9), and then cycled twice in this buffer. The resulting pellet was then resuspended in 1 ml of 0.5 M PMEG on ice for 30 min, and then centrifuged at 250,000 g for 3 h. 50 μl aliquots of the high speed supernatant were then used immediately in hook decoration experiments or frozen in liquid N$_2$ for future use.

Hook Decoration of T. brucei Cytoskeletons

10 ml of mid-log phase trypanosomes (4–5 × 10^6 cells/ml) were harvested by centrifugation (250 g, 10 min). The cells were gently extracted by resuspending them in 1% NP-40 in PEME (1 mM MgCl$_2$, 1 mM EDTA, 1 mM GTP, 2 mM EGTA, 0.1 M Pipes, pH 6.9) on ice for 10 min. The cytoskeletons were collected by centrifugation at 250 g for 30 min. The cytoskeleton pellet was then washed by gentle resuspension in PEME and

centrifuged at 250 g for 10 min. This pellet was resuspended in polymerization buffer, 0.5 M PMEG plus 5% DMSO, containing oligomer depleted microtubule protein at a final concentration of 2.5 mg/ml. The mixture was then incubated at 37°C for 60–120 min. After hook decoration had occurred, the cytoskeletons were fixed in 2.5% glutaraldehyde in PEME containing 1% tannic acid at room temperature for 60 min, and then in 1% Osmium tetroxide in PEME at room temperature for 45 min. After washing in PEME, they were stained in 2% Magnesium uranyl acetate overnight at 4°C, embedded in Spurr's resin, sectioned, stained, and viewed using a Philips 400 transmission electron microscope. Electron micrographs of forty hook decorated cytoskeletons were used for the microtubule polarity studies.

Seeding of Whole Mount Cytoskeletons

A sheep brain Hp$_2$ pellet was solubilized in 3 ml PEM (0.1 m Pipes, 1 mM EDTA, 1 mM MgSo$_4$) at 4°C, and diluted 20-fold to a final concentration of 0.36 mg/ml, this being the concentration of microtubule protein previously determined as being incapable of self assembly in vitro (data not shown). The solution was brought to 2 mM GTP, aliquoted onto clean strips of Nescofilm, and placed in a moist atmosphere at 4°C (15–20-μl aliquots). Trypanosome cytoskeletons were prepared by extraction on carbon coated, formvar grids with 1% Triton X-100 in PEM for 60–90 s at room temperature (longer extractions reduce seeding capacity of cytoskeletons). The grids with the extracted, whole mount cytoskeletons were floated onto the microtubule protein mixture covered to prevent evaporation and warmed to 37°C for 30 min. Sample grids were removed at 5, 10, 15, 20, and 30 min to assess assembly onto cytoskeletons. Cytoskeletons were fixed in 2.5% glutaraldehyde in PEM and stained with saturated uranyl acetate in water or 0.7% aurothioglucose.

Antibodies and Immunofluorescence

Immunofluorescence labeling of trypanosomes was carried out as previously described by Sherwin et al. (1987). YL 1/2, a monoclonal antibody specific for tyrosinated α-tubulin was the kind gift of John Kilmartin (Kilmartin et al., 1982).

Drug Treatment and Processing of the Cells

Trypanosomes were grown to mid-log phase, and then inoculated with the desired concentration of rhizoxin, diluted in DMSO (dimethyl-sulphoxide, BDH). The final amount of DMSO in culture was 0.05%. Rhizoxin was kindly donated by Shigeo Iwasaki (Institute of Applied Microbiology, The University of Tokyo, Bunkyo-ku, Tokyo, Japan). DAPI staining of 8-h drug-treated cells was carried out as described by Sherwin et al. (1987), but the cells were not washed in PBS, instead they were dried directly on poly-L-lysine–coated slides (to avoid selective attachment of any of the drug-produced forms). Also, fixation was done in 3.8% paraformaldehyde for 15 min, followed by −20°C methanol.

Whole mount cytoskeletons were prepared as described by Sherwin and Gull (1989a), but the cells were taken from the medium and were left to settle on grids, without washing in PBS, to avoid further disruption of the fragile drug-treated cytoskeletons.

DNA Fluorescence Quantitation

The relative DNA content of individual nuclei was estimated by scoring the DAPI fluorescence intensity from each nucleus, using a CCD (charged-coupled device) camera (type C3077, Hamamatsu). Images were recorded and the fluorescence was estimated using the Fluoanalysis image processing system (Improvision, Coventry, UK), running on a Macintosh Quadra 950 computer.

Results

Organelle Positions and Cytoskeleton Growth

We have shown previously that it is possible to use transmission electron microscopy of negatively stained, detergent-extracted cytoskeletons of *T. brucei* to reveal many features of the complete cytoskeleton (Sherwin and Gull, 1989a). The overall cell shape, together with indicators such as the

remnants of the nucleus (or nuclei), presence and length of the new flagellum, position of basal bodies, and other organelles allows an accurate positioning of the cells within a particular temporal window of the cell cycle. Examination of cytoskeletons by this method allows a single classification into four categories: 1F1N; cells possessing a single flagellum and a single nucleus (Fig. 1 a). 2F1N; cells possessing two flagella of any length and a single nucleus (Fig. 1 b). M; cells possessing two flagella of any length and a mitotic nucleus (Fig. 1 c). 2F2N; postmitotic cells possessing two flagella and two nuclei (Fig. 1 d). These figures are representative of many images of cytoskeletons produced from cells in an asynchronous culture. Such images were chosen at random in order not to bias the natural balance of cell types present within an exponentially growing culture. Each image of a cytoskeleton was examined and a catalogue of parameters was measured to produce a description of the position of each organelle within the cell relative to the overall

cell and flagellar lengths. The positional descriptors of 180 images of detergent-extracted negatively stained cytoskeletons were measured. These parameters were: (A) cell body length; (B) distance from the anterior end of the cell body to the center of the most anterior daughter nucleus; (C) distance from the center of the most anterior daughter nucleus to the basal body (BB1) subtending the mature flagellum; (D) distance from BB1 to the new basal body (BB2); and (E) distance from BB2 to the posterior end of the cell body. The relationships of these parameters in the four different cell categories (representative of discrete phases of the cell cycle) described above are seen in the diagram (Fig. 2 a). However, each of these morphological categories will contain cells at varying positions within that cell cycle window. Thus we also sought a constant parameter against which to standardize other variables. The mature flagellum apparently presents such a stable and easily identifiable parameter as a cell will begin the cell cycle with an already mature flagellum which
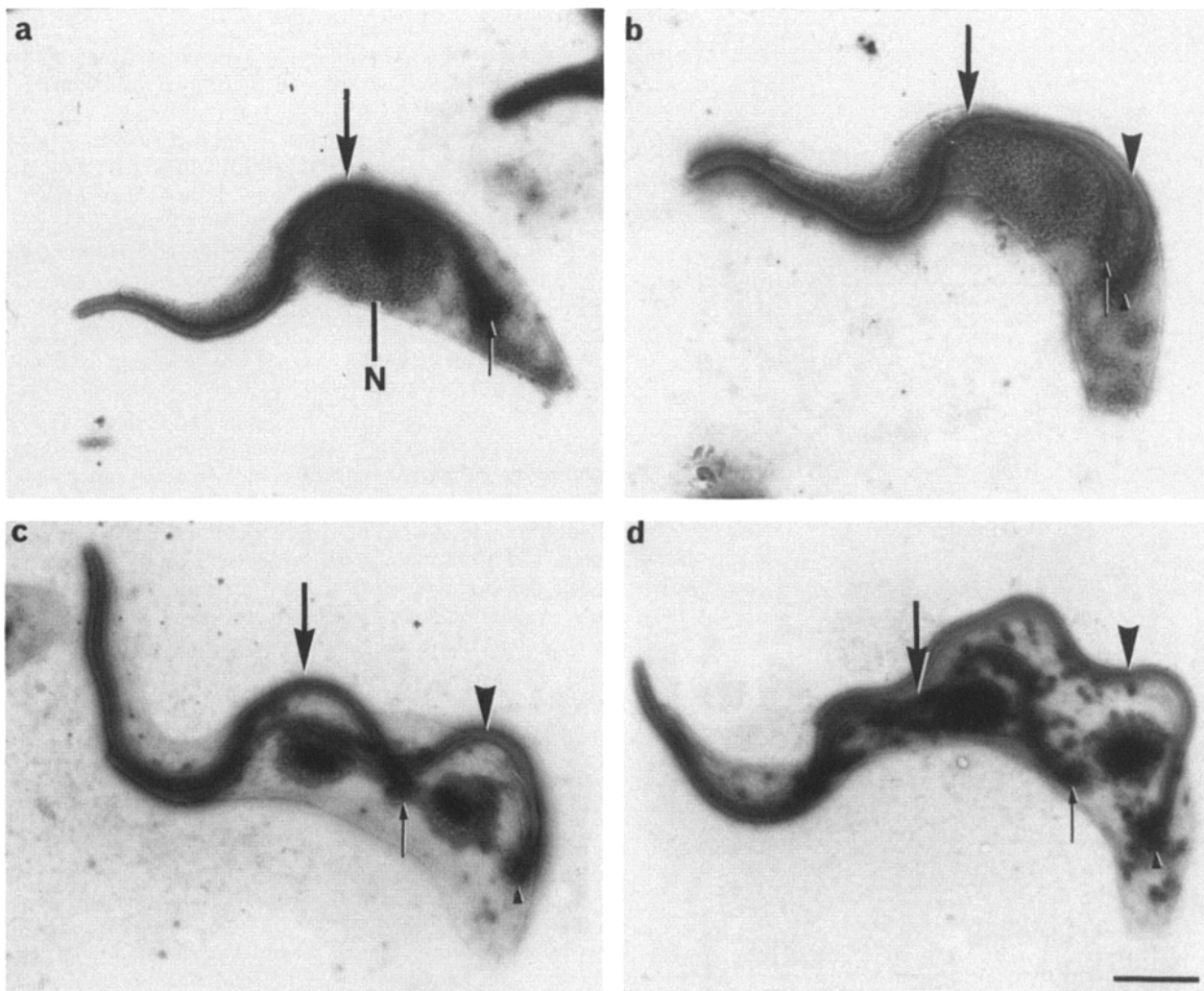
Figure 1. Transmission electron micrographs of negatively stained cytoskeletons of detergent extracted cells illustrating the four major cell cycle stages of T. brucei. (a) Cytoskeleton of a cell in the G1 phase of the cell cycle; this cell has a single flagellum (thick arrow), nucleus (N), and basal body (thin arrow). b shows the cytoskeleton of a cell further on in the cell cycle than (a) in which a second flagellum (arrowhead) has developed and the basal bodies (thin arrow old basal body, small arrowhead new basal body) are beginning to separate. (c) Cytoskeleton of a trypanosome with mitotic spindle and well separated basal bodies. d shows a postmitotic cell cytoskeleton in which one of the daughter nuclei has moved towards the posterior of the cell into the gap created by the separated basal bodies. Bar, (a–d) 3.5 µm.

a

A

1F1N

2F1N

M

2F2N

B — C — D — E

ANTERIOR   POSTERIOR

b

1F1N   2F1N   M   2F2N

Ratio : Positional descriptor / mature flagellum length

1.20
1.00
0.80
0.60
0.40
0.20
0.00

0.00   0.20   0.40   0.60   0.80   1.00
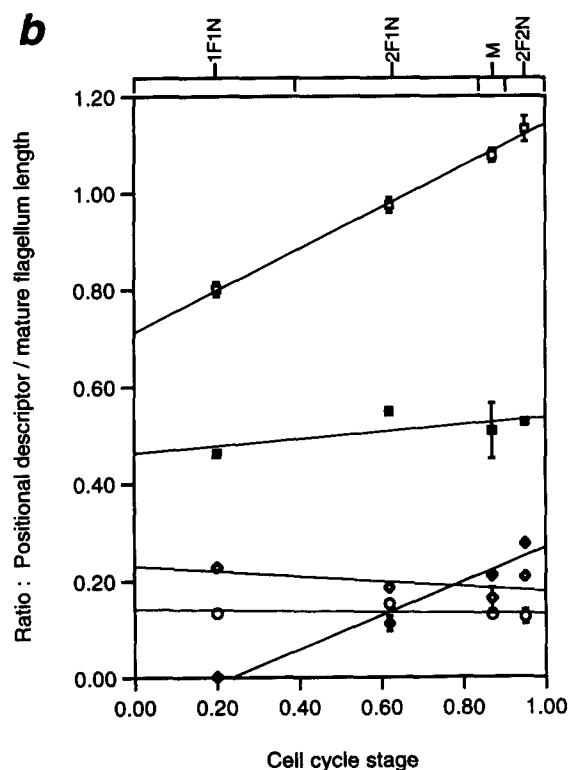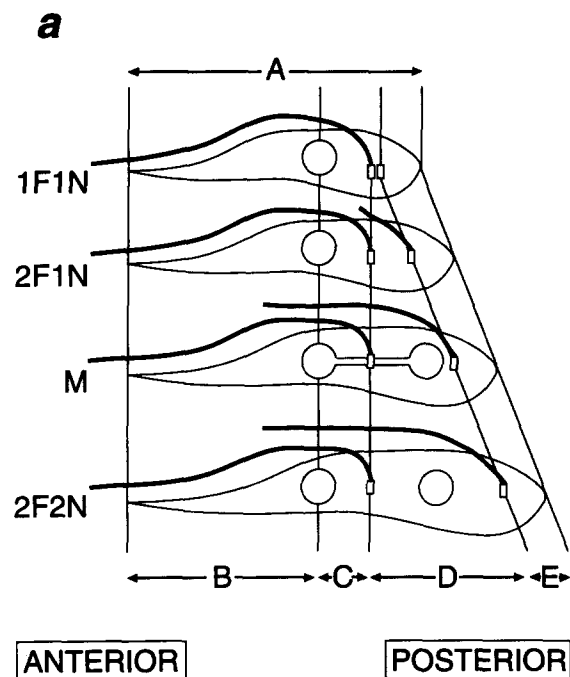
Cell cycle stage

Figure 2. (a) *T. brucei* procyclic form growth parameters derived from analysis of cells at various parts of the cell cycle. Positional descriptors as defined and illustrated schematically in Fig. 2 *a* are plotted quantitatively in Fig. 2 *b* against the average position of that particular cell cycle stage on a procyclic cell map. We measured organelle positional descriptors defined as follows: *A*, cell body length; *B*, distance from the anterior end of the cell body to the center of the anterior nucleus; *C*, distance from the center of the an-

is unlikely to change. Measurements of this parameter on all the cytoskeletons that we examined shows that this is indeed the case. The average mature flagellum length in any cell cycle stage remained essentially at a constant length of 22.3 μm ± 0.1 (mean ± S.E.). Thus, for every individual cytoskeleton measured, the position of each organelle was normalized by expressing the value relative to the length of the mature flagellum of that cell. This normalization then allows these measurements of position parameters for each organelle to be averaged and discussed within each of the four cell categories (1F1N, 2F1N, M, and 2F2N) described above. In turn, any changes in organelle position with progression through the cell cycle can then be determined and compared with the growth of the overall cell and microtubule cytoskeleton.

The average length of the cytoskeletons of the population of cells in the first stage of the cell cycle (1F1N) is 18.9 μm ± 0.3 (mean ± S.E.), (0.84 of the mature flagellum length), this length increases with progression through the cell cycle until it reaches 24.9 μm, ±0.3 (mean ± S.E), (1.16 of the mature flagellum length), just before cytokinesis (2F2N). Thus there is an overall increase in cell body length of ~6 μm during the cell cycle, and so growth of the trypanosome cell is not achieved simply by a doubling in length followed by cytokinesis at a midpoint. The rather thin, elongate nature of the cell together with the asymmetry of major organelle positioning (kinetoplast, basal-body, nucleus; posterior to anterior), dictates that the cell orchestrates its division by a small change in length and a helical, biased-longitudinal cleavage (Sherwin and Gull, 1989*a*). Given the linear arrangement of the major organelles and that the cell extends only ~6 μm (a 32% length increase) during the cell cycle, it is clear that in addition to duplication of the main organelles there has to be a precise structural repositioning. Essentially, in order for the cleavage plane to bisect the cell, it proceeds longitudinally (from the anterior to the posterior of the cell) while separating the duplicated organelles.

Considering the 6-μm overall length increase, we asked whether the distances involved in all organelle movements are in proportion or whether there is differential positioning of certain organelles at particular points in the cell cycle. Analysis of all of the measured parameters outlined above, reveals that the distance B—center of the most anterior nucleus to anterior end of the cell—apparently remains constant throughout the cell cycle (Fig. 2, *a* and *b*). This finding is also true for the distance C from the most anterior nucleus to the basal body subtending the mature flagellum and for the distance E from the new basal body to the posterior end of

terior nucleus to the basal body (BB1) subtending the mature flagellum; *D*, distance from BB1 to the new basal body (BB2); *E*, distance from BB2 to the posterior end of the cell body. (*b*) A quantitative illustration of the changes in the above positional descriptors plotted against a procyclic cell cycle map. Parameters measured are represented as follows: (□) cell body length, (■) distance from the anterior end of the cell body to the center of the anterior nucleus, (◇) distance from the center of the anterior nucleus to the basal body (BB1) subtending the mature flagellum, (◆) distance from BB1 to the new basal body (BB2), (○) distance from BB2 to the posterior end of the cell body. The distance between each organelle of interest was divided by the mature flagellar length and this ratio was plotted against the average timing of the cell cycle stage in a unit cell cycle map (Woodward and Gull, 1990). Bars represent standard error of the mean.

the cell. In marked contrast to these constant parameters, the distance D between the two basal bodies increases significantly with progression through the cell cycle. Moreover, comparison of the increase in cell body length with this inter-basal body distance reveals the slope of the plots to be almost identical for these two parameters (Fig. 2 b). This suggests that it is the increase in inter-basal body distance which accounts for the increase in cell body length, both in timing and extent.

The precision with which these events are coordinated within the cell cycle is also illustrated by the analysis of the relationship between growth in length of the new flagellum and movement apart of the basal bodies (Fig. 3). This data indicates that the new flagellum increases in length during the full distance (6 μm) of basal body separation. Examination of the data describing this process shows it to have biphasic characteristics with an inflexion at a particular inter-basal body distance. The new:old flagellum length ratio increases rapidly up to the point when the basal bodies are ~2 μm apart. However, after that distance the increase in the length of the new flagellum appears to occur at a slower rate. This inflexion in the kinetics data in Fig. 3 could occur by a reduction in the rate of new flagellum elongation, by an increase in the rate of basal body separation, or by a combination of these two factors. Whatever the reason, the outcome is such that the plot allows us to recognize a change in the microtubule-mediated morphogenesis of the cell at an inter-basal body distance of 2 μm. This reveals a new morphological event marker for an intrinsic, cryptic transition point in the trypanosome cell cycle.

Microtubule Polarity in the Trypanosome Cell

The above analysis provides evidence for specific organelle positioning within the procyclic cell cycle, together with coordination of organelle movements and growth of the cell and new flagellum. These phenomena appear likely to be linked to the method of duplication of the cytoskeletal corset of microtubules. Polarity of microtubules provides an inherent definition of the relative dynamics at each end of the microtubule. We have applied three different tests in order to determine the polarity of these sub-pellicular microtubules.

First, we have used the well established technique of hook decoration (Heidemann and McIntosh, 1980; Heidemann, 1991; Burton and Page, 1981; Heidemann et al., 1984; Baas et al., 1988). Detergent-extracted cytoskeletons of *T. brucei* were incubated with an exogenous solution of tubulin under conditions designed to decorate microtubules with protofilament hooks when viewed in transverse section by transmission electron microscopy. We used the organized architecture of the basal body and axoneme to establish cytoskeleton orientation. In this approach we needed to decorate a structure that is already highly cross-linked. We have established conditions that maintain the structure of the cytoskeleton yet allow labeling of large numbers of microtubules in the complex. Furthermore, due to the helical arrangement of microtubules in the cytoskeleton not all the decorated microtubules can be observed in the same plane of focus at any one time. Therefore, each cytoskeleton profile was observed using a tilting stage to collect microtubule profiles from each of 40 individual cytoskeletons (Fig. 4, a and b). In quantifying the microtubule polarity data, we found that
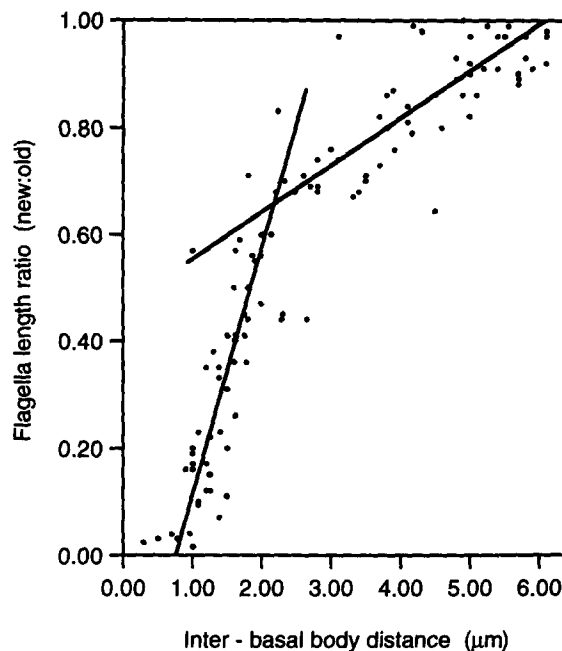

Figure 3. The relationship between inter-basal body distance and flagella growth. The inter-basal body distance and flagella lengths of 110 detergent-extracted negatively stained 2F2N cytoskeletons were measured. Inter-basal body distance was then plotted against flagella length ratio (new:old). The plot reveals an important change in the development of this relationship at an inter-basal body distance of ~2 μm. This morphological cell cycle marker occurs in G2 of the cell cycle.

in cytoskeletons viewed from the posterior towards the anterior 93% of all decorated microtubules have a clockwise hook curvature (1,200 from a total number of decorated microtubules of 1,298) showing an overall polarity of + end at the posterior end of the cell. This is the opposite polarity to that of the axoneme microtubules of the same cytoskeletons. Examination of serial sections of decorated cytoskeletons showed that microtubules exhibit a constant labeling pattern and consequent polarity along their length. We did not detect any indication of a variation of this polarity for cells in different parts of the cell cycle or within different sections of the same cytoskeleton.

The second method by which the polarity of the trypanosome cytoskeleton was tested was seeding from existing ends of microtubules. We incubated cytoskeletons with a solution of brain microtubule protein such that tubulin was assembled onto the ends of the existing microtubules. Whole cytoskeletons (110 in total) were analyzed by negative stain transmission electron microscopy. A very large plume of microtubules was seen to extend from the posterior end of the cytoskeletons, while there was little growth of microtubules at the anterior end (data not shown). Time course experiments revealed that more microtubules polymerized onto the posterior end than onto the anterior end such that after 15 min the posterior end nucleated around 18 microtubules vs 3 at the anterior. Fig. 4 c illustrates that the rate of microtubule assembly is four to five times greater onto the posterior end of the cytoskeleton than onto the anterior end. Clearly, both of these parameters (length and number) indicate that + ends of the sub-pellicular microtubules are located at the
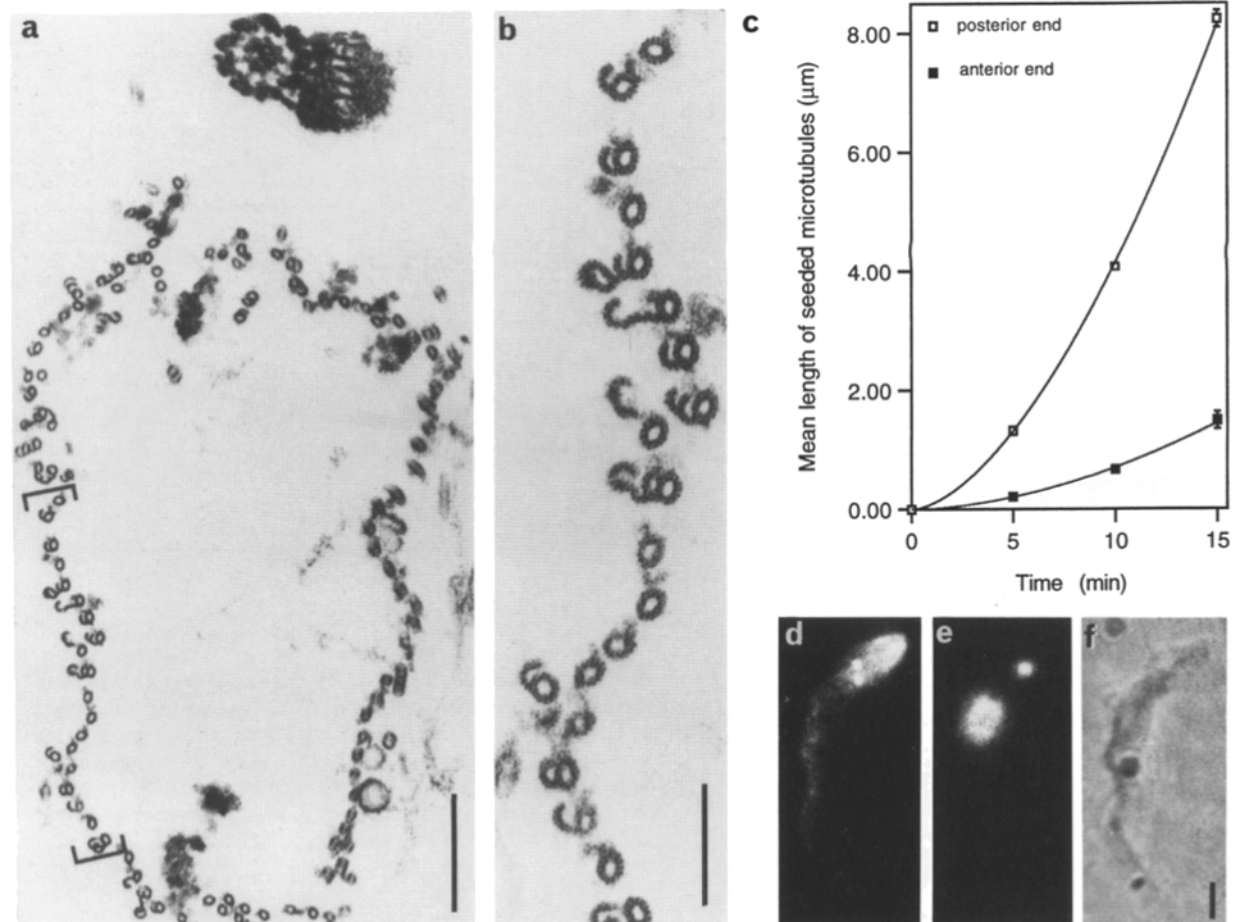
Figure 4. Illustrations of microtubule polarity within the cytoskeleton of *T. brucei* after using three independent assays. (*a*) Transmission electron micrograph of a hook decorated cytoskeleton. (*b*) Higher magnification of the region between square brackets in Fig. 4 *a*, showing that all hooks have grown in a clockwise direction. (*c*) Microtubule protein seeding onto detergent-extracted cytoskeletons. Bars represent standard error. (*d*) Immunofluorescence, (*e*) DAPI, (*f*) phase contrast micrographs of *T. brucei* cells immunolabeled with the YL 1/2 rat monoclonal antibody which recognizes tryosinated α-tubulin. Bars: (*a*) 0.25 μm; (*b*) 0.1 μm; (*d–f*) 2 μm.

posterior end of the cytoskeleton. The rate of brain tubulin assembly onto cytoskeletal microtubules (plus and minus ends) compares favorably with the rate of assembly onto flagellar "seeds" (Bergen et al., 1980).

Both of the above assays provide direct tests of polarity. In a third assay we have looked at the overall pattern of endogenous tubulin incorporation in different zones of the cytoskeleton by detecting the location of tyrosinated α-tubulin. We have previously shown in *T. brucei*, that tyrosinated α-tubulin acts as a marker for newly assembled microtubules (Sherwin et al., 1987; Sherwin and Gull, 1989*b*). These experiments were performed using the monoclonal antibody, YL 1/2, which recognizes *T. brucei* α-tubulin only if the carboxy-terminal tyrosine is in place (Kilmartin et al., 1982; Wehland et al., 1984). Since detyrosination is a postassembly modification, this monoclonal has the ability to locate sites of recent tubulin addition to microtubule ends. When the cytoskeletons of cells in the early part of the cell cycle are labeled with this monoclonal antibody and viewed by fluorescence microscopy (Fig. 4 *d*), high levels of fluorescence were observed in the posterior third while the rest of the cytoskeleton remained essentially unstained (see also Sherwin et al., 1987; Sherwin and Gull, 1989*b*). We inter-

pret this pattern as indicative of the larger tyrosinated, new tubulin mass associated with the fast growing, + ends of the sub-pellicular microtubules.

Thus, three independent tests of microtubule polarity show that the microtubules of the sub-pellicular corset have the same polarity, that is with their + ends at the posterior end of the cell.

Microtubule-mediated Events and the Cell Cycle

There are a number of events in the cell cycle which directly involve microtubule-mediated processes. These include growth of the cell by addition to the sub-pellicular array, movement apart of basal bodies which is in itself intimately linked with the preceding process (Robinson and Gull, 1991), growth of the new flagellum, mitosis, and cytokinesis (Woodward and Gull, 1990). This raises the question of the relationship between such periodic events. We have been able to use different concentrations of the antimicrotubule agent rhizoxin to affect particular combinations of the above microtubule-mediated processes. In essence, flagellum growth appears least sensitive to drug inhibition while mitosis and sub-pellicular microtubule events are compro-
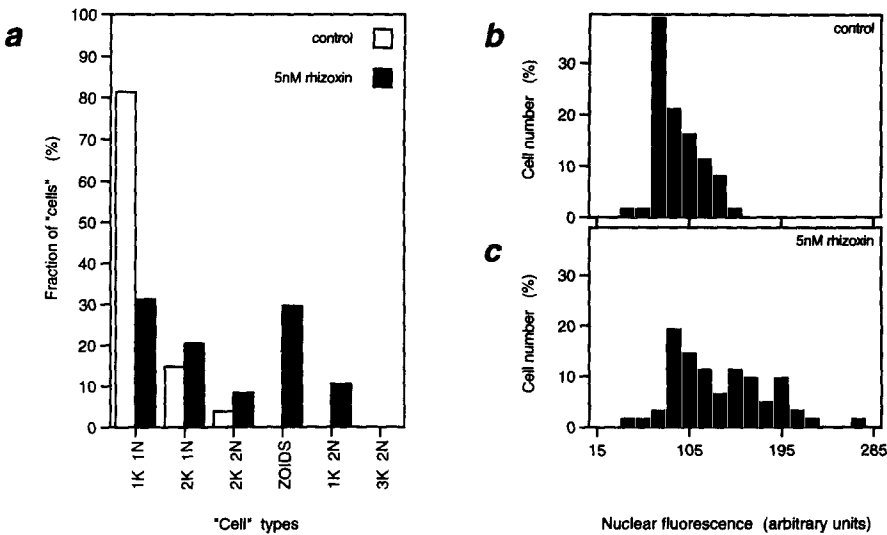
Figure 5. (*a*) *T. brucei* culture form cell types of control and rhizoxin-treated (5 nM rhizoxin, 8 h) cultures. Proportions of cell types in control cultures are classified in cell cycle position by their possession of replicated and segregated kinetoplasts and nuclei. In the presence of rhizoxin these proportions change dramatically and novel forms (zoids [1K0N] and 1K2N) appear, which result from aberrant divisions. (*b*) Nuclear DNA content of 1K1N cells of control, measured by quantitative DAPI fluorescence. 1K1N cells are in the G1 or S phase of the cell cycle. (*c*) 1K1N cells of rhizoxin-treated cultures have higher DNA contents, indicating possession of replicated yet unsegregated nuclei.

mised by low concentrations of rhizoxin. When we treated an exponentially growing culture of trypanosomes with a low (5 nM) concentration of rhizoxin, we noticed that cells accumulate with nuclear and kinetoplast configurations that vary remarkably from the controls (Fig. 5). In untreated cultures, cells early in the cell cycle possess a single kinetoplast and a single nucleus, these cells are termed 1K1N. As the cell cycle progresses the kinetoplast replicates and segregates before the nucleus to produce a 2K1N cell. Cells then enter mitosis, the outcome of which is a 2K2N cell. Finally cytokinesis occurs, cleaving the cell into two 1K1N siblings. Cytological analysis, using DAPI staining of the cells from cultures treated with 5 nM rhizoxin for 8 h (which approximates to the duration of one cell cycle period) (Woodward and Gull, 1990), revealed a remarkable phenomenon. A very large percentage of the "cells" turn out to be anucleate and yet have a kinetoplast (1K0N) (Fig. 5 and Fig. 6, *b* and *b'*). Therefore, these cells are in fact cytoplasts. They are flagellated and motile and we have termed them zoids. The fact that 30% of the cells in the treated culture are zoids shows formally that cytokinesis does occur, albeit with an unusual outcome at this drug concentration. In comparison to controls the number of cells with a single kinetoplast and single nucleus (1K1N) are massively reduced in the rhizoxin culture and moreover there is an appearance of an unusual class of cells that have a single kinetoplast and two nuclei (1K2N). The reduced number of 1K1N cells after rhizoxin treatment is a consequence of the division that produced the zoids. The 1K2N cells must represent the other sibling of the division that was compromised in nuclear segregation and produced a zoid (1K0N). However, after approximately one cell cycle period in the asynchronous culture (Fig. 5), there were approximately twice as many zoids as 1K2N cells. Thus, an almost equal number of zoids must also have been produced from another type of division. The other sibling, resulting from this other division, is most likely to be the 1K1N cells found in the rhizoxin culture. In this division cells apparently failed to complete mitosis, and then these 2K1N cells continued on to complete the cell cycle, undergoing cytokinesis to form a zoid (1K0N) and a cell (1K1N). Such 1K1N cells should, in contrast to control 1K1N cells, possess a repli-

cated but unsegregated nuclear genome. This is indeed the case. We used quantitative fluorescence methods to measure the DNA content of individual nuclei in the 1K1N cells within control and rhizoxin-treated cultures. After an 8-h rhizoxin treatment, a large proportion of cells within the 1K1N population show elevated levels of nuclear DNA (Fig. 5, *b* and *c*).

Axis and Polarity of Cytokinesis

We now asked whether there was any particular spatial pattern involved in zoid production? Cells treated with a higher concentration of rhizoxin have many of their microtubule processes inhibited and it is easier to accumulate cells blocked in the process of zoid production. Examination of cytoskeletons of such cells with a zoid still attached to the main cell body show that the zoid arises from the posterior portion of the cell and inherits the shorter, new flagellum (Fig. 6 *c*). Zoids almost always have a single flagellum and kinetoplast and so cytokinesis appears to be occurring in a relatively precise manner in these drug-treated cultures with respect to cortical axis and parameters. Is there some mechanism that links or defines the initiation and direction of the cleavage furrow within the sub-pellicular corset of microtubules? In this context we also noted that zoids can vary somewhat in size, in particular some zoids had a length that was much shorter than the normal division (birth) length of a trypanosome cell. It is possible that this may be a consequence of the position of the particular parental cell in the cell cycle when the drug was added. However, in general terms the length of the body of the zoid was usually in proportion to the length of its flagellum (Fig. 6, *d*, *e*, and *f*). This suggests that the position of cleavage furrow initiation in cytokinesis is defined with respect to the distal end of the new flagellum that is always inherited by the zoid. This would suggest some link between the cytoplasmic microtubule corset and the flagellum and we discuss below a possible role for the flagellar attachment zone (FAZ)[1] in this process.

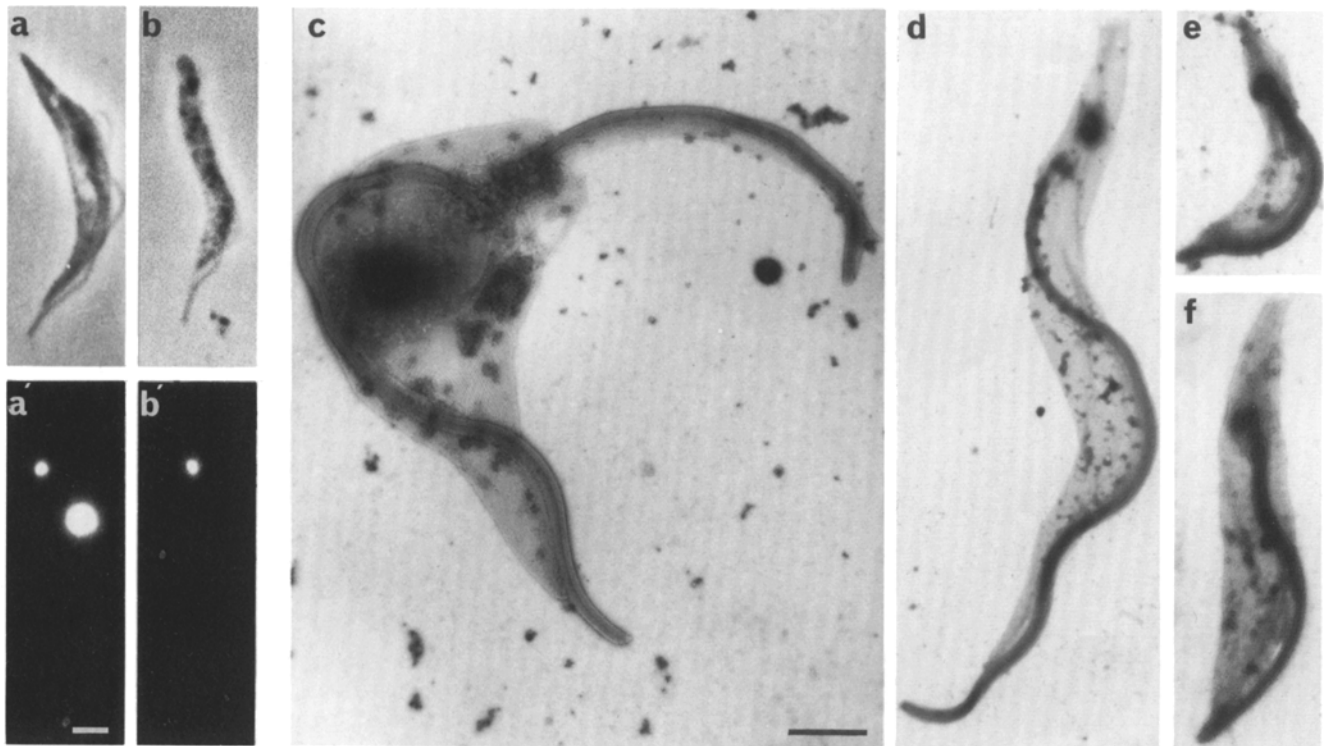1. *Abbreviation used in this paper:* FAZ, flagellar attachment zone.

Figure 6. (*a* and *b*) Phase contrast images and (*a'* and *b'*) corresponding DAPI fluorescence images of *T. brucei* culture form cell types, (*a'*) control 1K1N cell and (*b'*) rhizoxin-induced zoid (1K0N) showing absence of nucleus. (*c–f*) Transmission electron micrographs of detergent-extracted negatively stained, abnormal cell types produced after cultures were incubated for 8 h with (*c*) 100 nM rhizoxin and (*d, e,* and *f*) 5 nM rhizoxin. *c* shows a cell inhibited in mitosis that is cleaving to produce a zoid; *d, e,* and *f* show cytoskeletons of zoids of different sizes. Bar: (*a–f*) 2 μm.

Discussion

The trypanosome cell exhibits a very pronounced polarity defined by the asymmetry of the position of organelles and cell shape. The major structural component of the trypanosome cell providing this shape asymmetry (broad end, posterior; narrow end, anterior) is the cortical array of microtubules. Individual microtubules will themselves have an intrinsic polarity and the question arises as to whether this influences the overall cell polarity. All three polarity assays show the + end of microtubules to be at the broad, posterior end of the cell. We have shown previously that addition of new microtubules into this sub-pellicular lattice occurs by intercalation between the existing microtubules (Sherwin and Gull, 1989*b*). We have speculated that this may be influenced by changes in the extensive intermicrotubule cross-links. However, whatever the molecular mechanism, this polarity information shows that the new cohort of microtubules laid down throughout the cell cycle maintains the uniform individual microtubule polarity. It appears possible that this polarity of individual microtubules has implications for the polymerization kinetics within the sub-pellicular array and so, in turn, contributes directly to the asymmetry of cell shape. Thus, in a situation where addition of subunits is possible at both ends of a microtubule one expects that in a given time period there will be more extension at the + end (Bergen and Borisy, 1980; Margolis and Wilson, 1978). In the trypanosome cell sub-pellicular array this means that there will be much more polymerization of in-dividual microtubules at the posterior end of the cell and also individual new microtubules will invade the interzones between existing microtubules. This bulk of polymerization in a local area may be a critical factor in providing the molecular mechanism that defines the posterior end of the cell as the broad end.

Our results also help to explain the discrete localization of a 28-kD microtubule-associated capping protein, Gb4, that is located only at the posterior end of the trypanosome cytoskeleton (Rindisbacher et al., 1993). Our finding that these sub-pellicular corset microtubules have their + ends at this cell position rather than in a random or anti-parallel polarity would explain how a + end capping protein would have a restricted location in the trypanosome cell.

Segregation of Organelles and a New Cell Cycle Event Marker

Our measurements show a clear correlation between the degree of overall cell extension and the increase in one particular positional indicator, the inter-basal body distance. We have shown previously that the movement apart of the basal bodies is a microtubule-mediated process and can be inhibited by the maytansine group of antimicrotubule agents. This movement apart of the basal bodies is a critical step in the cell cycle since it is this process that is responsible for segregation of the kinetoplast DNA (Robinson and Gull, 1991). Examination of the cell cycle of *T. brucei* reveals that there is a discrete S phase for both the mitochondrial and nu-

clear DNA. Mitochondrial genome segregation occurs in the middle of the nuclear G2 period, by the movement apart of the basal bodies. Mitosis occurs when kinetoplast segregation is complete, at an inter-basal body distance of ∿4.5 μm. The present measurements show that flagellum growth and movement apart of the basal bodies occur simultaneously. Our results reveal a specific change in the relationships of these two events at an inter-basal body distance of 2 μm. An inter-basal body distance of 2 μm corresponds to a point when the kinetoplast has an elongate or dumb-bell shaped appearance. Therefore, a likely possibility is that it is at this point in the cell cycle that the replicated kinetoplast DNA complex has become "disentangled" and the daughter kinetoplasts can be segregated by the basal bodies (Robinson and Gull, 1991). Hence, the rate of basal body segregation may well increase after this point. This analysis, however, reveals a new and useful morphological event marker for a cryptic transition point (the ct point) in G2 of the trypanosome cell cycle. It is likely that the ct point occurs around very late D or in the early A phase of the "kinetoplast cell cycle" as defined by Woodward and Gull (1990).

Basal body segregation separates the replicated kinetoplast before segregation of the nuclear genome is initiated and so opens up a gap which, after the forthcoming mitosis, will be occupied by one of the daughter nuclei. The trypanosome cell is vermiform in profile, with an essentially linear arrangement of organelles and so, in order to define a single axis for division, it has to move one of the replicated organelles around another one. The solution to this geometric problem is to segregate the mitochondrion/kinetoplast/basal body/flagellum complex as one, and then to move one of the daughter nuclei around one of these complexes.

Nuclear Positioning and Mitosis

Our analysis of the positional descriptors in the trypanosome cell also indicate that the nucleus has a very defined position, however there is little information on the nature of any anchoring system. There are no microtubules in the cytoplasm other than the sub-pellicular array and electron micrographs show that the nucleus does not touch this cortical array of microtubules. This raises the possibility of another filament system providing nuclear positioning and anchorage. There is little structural or biochemical evidence for such a system. Trypanosomes contain actin as evidenced by the possession of transcribed actin genes (BenAmar et al., 1988) but there is little reliable evidence for the location of the non-abundant actin protein. In spite of the lack of evidence for such an anchoring system, we have made the interesting observation that the anterior nucleus maintains its position relative to the anterior of the cell and the anterior basal body while the cell elongates and even during mitosis. These measurements suggest an explanation of why the intranuclear mitotic spindle straddles the axis of the old flagellum. A mechanism that anchors the nucleus in relation to the anterior basal body, while the posterior basal body moved away in concert with cell elongation, would set up the correct spatial positioning ready for "delivery" of a daughter nucleus into the gap after mitosis. In contrast to many organisms it is important to note that the basal bodies are not at the poles of the trypanosome spindle. The fact that the nuclear envelope remains intact throughout mitosis could implicate the envelope as the site of this postulated nuclear anchoring system. We do not infer

by the above that the spindle itself has a unidirectional force, merely that if the anchoring mechanism for the anterior daughter nucleus is maintained at, and beyond, metaphase, then this may be able to account for movement of the posterior daughter nucleus into the gap between the basal bodies.

We have little information of the mechanics of spindle movement in these cells. However, anaphase A and chromosome segregation, as defined by DAPI staining, takes place before movement of the daughter nucleus into the gap (Sherwin and Gull, 1989a). Therefore, the positioning of the posterior daughter nucleus into the gap is essentially an anaphase B/spindle elongation process. In this way, mitosis in trypanosome cells achieves both chromosome segregation and daughter nucleus positioning. T. brucei cells possess a large number of differently sized chromosomes (Van der Ploeg et al., 1989) and it is unclear whether the abundant, very small chromosomes are separated by the microtubule mitotic spindle. In such a case the nuclear anchoring and movement system postulated above may have implications for the segregation fidelity of such chromosomes via a nuclear matrix form of attachment.

Cytokinesis Checkpoints

The rhizoxin-treated cultures reveal that cells which fail to undergo mitosis or fail the later stages of correct nuclear positioning can still go on and undertake cytokinesis. In both cases cytokinesis results in formation of a zoid (1K0N) and another sibling that is either 1K2N or 1K1N. The particular type of division sibling to a zoid may well be dependent on the relative position of the parent cell within the cell cycle at the time of drug addition.

Interestingly, Sullivan and Huffaker (1992) have described a S. cerevisiae mutant (tub2-401) which fails to elaborate astral microtubules but can complete both mitosis and cytokinesis. Under specific conditions, cells of this mutant produce on division a cell with two nuclei and a cell minus a nucleus. These studies show that cell cycle progression does not depend on either the location or orientation of the spindle. These results are analogous to our findings of the division that produces the zoid (1K0N) and the 1K2N cell in the rhizoxin-treated T. brucei cultures. Although having apparently completed chromosome segregation, these cells have failed the late mitotic event of correct positioning of their daughter nuclei. However, they still go on and complete cytokinesis akin to the tub2-401 S. cerevisiae cells. To account for the number of zoids produced in these cultures we must consider that some are born from divisions of a cell that has failed both the chromosome segregation and nuclear positioning portions of mitosis and possesses a single nucleus. This cell (2K1N) would then cleave into a zoid (1K0N) and a 1K1N cell. In many eukaryotes the use of drugs or mutations to disrupt microtubule-mediated events often blocks progression through mitosis and, as a consequence, cytokinesis, indicating the presence of a cell cycle checkpoint involving microtubules. Taken with our description of the central feature of mitochondrial genome/basal body segregation in the earlier portion of the T. brucei cell cycle (Robinson and Gull, 1991), it is possible that the completion of cytokinesis in trypanosome cells that have failed mitosis is a reflection of the existence of novel organization of dependency relationships which lead to cell cycle checkpoints in these

cells. This may not be so surprising in such an ancient eukaryote, which in essence has a two genome segregation problem—needing to coordinate cytokinesis with the temporally separate phenomena of mitochondrial genome segregation and mitosis.

Axis and Polarity of Cytokinesis

Examination of cytoskeletons of *T. brucei* shows that cleavage of cytokinesis starts at the anterior end and follows a helical anterior to posterior path up the cell (Sherwin and Gull, 1989a). Cleavage must obviously take place between the old and new flagellum, but there has been little evidence for the nature of the events/structures that might set the site of cleavage furrow initiation. The fact that low concentrations of this drug produce anucleate zoids indicates that cytokinesis does not depend absolutely on the correct positioning of the nuclei. Moreover, zoids have different sizes and yet always have a flagellum (the new flagellum). Therefore, the choice of cleavage site must be more intensely influenced by inclusion of a segregated posterior basal body/new flagellum than by a daughter nucleus. Additionally, our drug studies show that the resulting zoids can have different sizes but that in general their flagellum length is in proportion to the body length, suggesting that the position of the original decision site for initiation of cleavage in the cytoplasmic corset of microtubules correlates with the length of the new flagellum. We postulate that one particular area of the cell, the flagellum attachment zone (FAZ), a filament system intimately associated with the sub-pellicular array of microtubules, could provide a structural correlate between the length and position of the new flagellum and the main growth of the cell body. Electron microscopy of cytoskeletons shows that this structure elongates at the same rate as the external flagellum. Moreover, we have confirmed this result by double label immunofluorescence of cells using monoclonal antibodies (Woods et al., 1989) that detect the flagellum and the FAZ filaments (data not shown). The FAZ is a complex area of the cell cortex but it is an invariant and precisely defined structure occurring at a unique site where, in a gap between two microtubules of the corset, a single filament runs down the cell. The flagellum membrane and the cell body membrane are intimately juxtaposed at this site and, in essence, the flagellum is cross-linked to this internal cytoplasmic filament. Moreover, at one side of this filament is a set of four microtubules that are differentiated by their close association with smooth endoplasmic reticulum and their intrinsic stability (Sherwin and Gull, 1989a). Thus we define the FAZ as comprising of both the filament system and the four microtubules that lie to its immediate left when viewed from the posterior of the cell (Sherwin and Gull, 1989a). Our hypothesis therefore is that it is this cytoplasmic FAZ area of the new flagellum that marks the position and direction of the cleavage furrow. Tests of these ideas involving specific destabilization of this complex may be possible in the future using molecular genetic approaches once we have information on genes encoding proteins specific to the FAZ.

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