**INTRODUCTION**

Co-ordination of cell cycle events is clearly necessary for cell survival and therefore it is essential that these events occur correctly, both sequentially and temporally. These events are driven by the activation and inactivation of cyclin dependent kinases and are monitored by specific signaling ‘checkpoints’ (Skibbens and Heiter, 1998; Mendenhall and Hodge, 1998). In yeasts, cell cycle checkpoints have been identified mainly in three phases: DNA synthesis is monitored by the DNA replication/damage checkpoints (Weinert and Hartwell, 1988; Rowley et al., 1992; Lydall and Weinert, 1995; Enoch and Norbury, 1995; Kostrub et al., 1998), mitosis by the spindle assembly checkpoint (Hoyt et al., 1991; Li and Murray, 1991; Skibbens and Heiter, 1998) and cell division by the cytokinesis checkpoint (Muhua et al., 1998).

The cell cycle of trypanosomes shows the same periodic nuclear events, G1, S, G2 and M phases, which characterise the normal eukaryotic cell cycle. In contrast to other eukaryotes, trypanosomes exhibit a periodic S phase for their unit mitochondrial genome, the kinetoplast (Steinert and Van Assel, 1967; Cosgrove and Skeen, 1970; Woodward and Gull, 1990). This network of DNA molecules is present as a single structure within the mitochondrion (Steinert et al., 1976; Ryan et al., 1988) and is located in a distended region, close to the base of the flagellum. The segregation of the kinetoplast, before mitosis, acts as an excellent additional cytological marker for the position of an individual cell in the cell cycle (Sherwin and Gull, 1989b; Woodward and Gull, 1990).

The trypanosome possesses a number of microtubule based structures including the flagellar axoneme, basal bodies, intranuclear mitotic spindle and a complex sub-pellicular corset of cytoplasmic microtubules. We have previously shown that microtubule mediated events occur throughout the cell cycle. Since cell shape and form are entirely dependent on the subpellicular microtubule corset, polymerisation of new microtubules is an essential prerequisite for cell growth within the cell cycle (Sherwin and Gull, 1989a; Robinson et al., 1995). Other major events such as kinetoplast segregation, basal body duplication, flagellar axoneme growth, mitosis and cytokinesis are also microtubule dependent processes involving microtubule polymerisation/depolymerisation. The order of these events and their duration in the cell cycle of *T. brucei* is known with a reasonable level of accuracy (Fig. 1); however, we have little idea of how these processes are related and regulated or how they relate to other cell cycle processes, such as the S phases of the nucleus (SN) and kinetoplast (Sk).

*T. brucei* is a diploid organism and so we cannot readily use selectional genetic approaches to study cell cycle regulatory processes. Also, there are no effective selection methods for synchronising trypanosomes. We have therefore adopted an approach involving drug inhibition of cell cycle events. Many eukaryotic microbes are resistant to the classical colchicine group of antimicrotubule agents, and this is a reflection of...
intrinsic differences in their tubulin molecules (Kilmartin, 1981; Quinlan et al., 1981; Werbovetz et al., 1999). We have found, however, that trypanosomes are also very resistant to the action of a vast family of benzimidazoles and related drugs (MacRae and Gull, 1990). Trypanosomes, and some species of Leishmania, show a degree of sensitivity to vinblistine (Ono and Nakabayashi, 1979; Grellier et al., 1999; Werbovetz et al., 1999). The vinblistine ‘binding site’ on tubulin is also shared by maytansine and, indeed, we have shown that both maytansine and ansamitocin are active against trypanosomes (Robinson and Gull, 1991). Another member of this group, rhizoxin, is the most potent antimicrotubule agent so far shown to be active against the trypanosomatids (Robinson et al., 1995; Ersfeld and Gull, 1997). Rhizoxin treatment of trypanosomes shows that, at certain concentrations, its antimicrotubule action results in bizarre effects on cells leading to the production of anucleate cytoplasts (Robinson et al., 1995).

The level of cross-talk between kinetoplast and nuclear S phases is unclear; however, at least this level must be a highly co-ordinated process. Cell division cycle checkpoints require successful completion of early acts before the initiation of later events controlling the progression of the cell cycle (Hartwell and Weinert, 1989; Murray, 1992; Skibbens and Heiter, 1998). The existence of two periodic S phases (SN and SK), two periodic genome segregation events (mitosis and kinetoplast segregation) and a single cytokinesis event, raises issues about the nature of the dependency relationships and cell cycle checkpoints that operate to control these events within the trypanosome cell cycle. Our drug inhibition experiments reveal novel features of cell cycle control in trypanosomes, which seem intrinsically linked to their requirement to replicate and segregate both a unit nuclear genome and a unit mitochondrial genome.

MATERIALS AND METHODS

Trypanosomes

Pro cyclic forms of Trypanosoma brucei brucei strain 427 were maintained in semi-defined medium (SDM 79), at 28°C (Brun and Schönengerber, 1979).

Drug treatment

For rhizoxin treatments, cultures were grown to mid-log phase (5×10⁶ cells/ml) and then inoculated with the desired concentration of rhizoxin, diluted in DMSO (dimethylsulphoxide; 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).

For aphidicolin treatments, mid-log phase cultures were inoculated with aphidicolin (Sigma) and diluted in 50% ethanol to a final concentration of 60 µM. Drug treatment

Monitoring growth of the trypanosome cultures

30 µl samples were taken in 2 hour intervals, during the 8 hour period of drug treatment. An equal volume of 2.5% glutaraldehyde was added to the sample and the cells were counted.

DNA staining of trypanosomes

DAPI (4,6-diamidino-2-phenylindole) staining of drug treated cells was carried out as described by Sherwin et al. (1987), except that the cells were not washed in PBS (phosphate-buffered saline), but dried directly on poly-L-lysine coated slides (to avoid selective attachment of drug-produced forms). Drug treated cells were fixed in 3.8% paraformaldehyde for 15 minutes, followed by −20°C methanol.

BrdU labelling of the cells and subsequent rhizoxin treatment

A mid-log phase culture was inoculated with 5-bromo-2-deoxyuridine (BrdU) and 2-deoxyctydite (Sigma) to a final concentration of 50 µM for 1 hour, at 28°C. Cells were washed twice in warm medium and the culture was divided into two portions: rhizoxin was added to one flask to a final concentration of 5 nM for 8 hours, and DMSO only was added to the second (control) flask. Samples destined for cell counts were taken every 2 hours, and for immunofluorescence, every hour during the 8 hour treatment period.

Immunofluorescence microscopy

Immunofluorescence of rhizoxin treated cells with anti-BrdU antibody was performed as described by Woodward and Gull (1990) except that cells were fixed in −20°C methanol. Cells were visualised using a Zeiss Axioskop. Cell ‘type’ counts were performed separately by two individuals. Approximately 800 cells were counted.

Immunofluorescence of aphidicolin-treated trypanosomes with monoclonal antibodies ROD1 and BBA4 (Woods et al., 1989) was performed according to Sherwin and Gull (1989b). The cells were visualised using a Leica DMRB microscope and digital images were recorded with a cooled slow scan CCD camera (Photometrics).

Fluorescence activated cell sorting (FACS) analysis

FACS analysis was carried out as described by Kooy et al. (1989) with the following modifications: cells were washed once in PBS, fixed in 30% PBS/70% methanol at −20°C for 24 hours and pelleted (1000 g, 10 minutes, 4°C). Cells were rehydrated in PBS for 5 minutes on ice and stained in PBS with 2 mM Hoechst 33258 (Sigma). Samples were analysed in a Becton Dickson FACS Vantage flow cytometer, excitation wavelength of 424 nm.

RESULTS

Rhizoxin and trypanosome growth and morphology

Rhizoxin is an extremely potent agent affecting growth of procyclic culture forms of T. brucei. 100 nM produces complete growth inhibition (Fig. 2) and even a 5 nM concentration partially inhibits growth (Fig. 2). However, these growth curves present an incomplete view of the effects of...
Cell cycle checkpoints in trypanosomes

Detailed examination of the rhizoxin treated cultures reveals the presence of bizarre and unusual ‘cell types’ (Fig. 3). Staining of control cultures with DAPI reveals three easily classified stages of the trypanosome cell cycle. Cells early in the cell cycle have a single kinetoplast (K) and nucleus (N) (1K1N) whilst later, after kinetoplast segregation, one can visualise 2K1N cells and later still, after mitosis, 2K2N cells (Fig. 3) (Sherwin and Gull, 1989b; Woodward and Gull, 1990).

After treatment with 5 nM rhizoxin, cells retain their characteristic shape but DAPI staining reveals a number of aberrant cell types. In addition to the usual 1K1N, 2K1N and 2K2N cells, there are many examples of cytoplasts, which we have termed ‘zoids’ (Robinson et al., 1995). Although these appear in phase contrast microscopy to be small cells (Fig. 3G) they are anucleate (Fig. 3G'). These 5 nM rhizoxin treated cultures also contain cells with a 1K2N complement (Fig. 3F). Also, a significant population of cells have unusual morphologies even though their nuclear/kinetoplast number appears normal (i.e. 2K1N or 2K2N). Examples of these are seen in Fig. 3D,E where one of the kinetoplasts in these cells is unusually distant from the other (Fig. 3D',E'). These cells appear to be in the process of division, the result of which would be to form a zoid (1K0N) and a 1K1N cell or a zoid and a 1K2N cell. Examination of the 100 nM rhizoxin treated cultures reveals more dramatic effects on cell shape. Cells become rounded and DAPI staining reveals unusual nuclear and kinetoplast complements in some cells (Fig. 3H-L). There are fewer zoids in these cultures but there are examples of 2K2N cells with unequal sized nuclei (Fig. 3L,L'), suggesting

![Fig. 2. Growth curves of T. brucei control and rhizoxin-treated cultures, during 8 hours of treatment, showing a concentration-dependent inhibition of growth by rhizoxin. Control cells are represented by open squares. 5 nM rhizoxin treated cells are represented by filled squares and 100 nM rhizoxin treated cells are represented by circles.](image)

![Fig. 3. Phase contrast and DAPI fluorescence micrographs of T. brucei cell types, defined by the number of kinetoplasts and nuclei in each cell. (A,A'-C,C') Controls showing the three major cell cycle stages in T. brucei. (D,D'-G,G') Aberrant cell types formed after 8 hour treatment with 5 nM rhizoxin; cell shape remains elongated and slender, but DAPI staining reveals aberrant numbers of kinetoplast and nuclear content. (D,D', E,E') Cells in cytokinesis. (F,F', G,G') Two aberrant cell types formed by this concentration of rhizoxin: a 1K2N cell and a zoid. (H,H',I-I', J,J', K,K', L,L') Cell types produced after an 8 hour treatment with 100 nM rhizoxin; the cell body has become rounded. The numbers of kinetoplasts and nuclei in I,I' and J,J' are aberrant (1K2N). (K,K', L,L') The two nuclei are unequal in size and in K,K' the relative position of the two kinetoplasts and the two nuclei is different to control cells (compare with C,C'). Bar, 3.5 μm.)](image)
that nuclear genome segregation has been severely compromised. Thus, given the nature of these bizarre cell forms in the rhizoxin treated cultures, the cell counts seen in Fig. 2 represent an underestimate of the effect of this drug.

A quantification of the cell types seen in control and drug treated cultures after 8 hours is presented in Fig. 4. At 8 hours of drug treatment in a low concentration of rhizoxin (5 nM) there is a steep reduction in the number of 1K1N cells in the population and a noticeable appearance of large numbers of zoids (1K0N) and of 1K2N cells (Fig. 4A). Since in normal cultures the kinetoplast divides before the nucleus, these 1K2N cells represent a cell type that is never present in a normal cell cycle. The pattern after 8 hours culture in 100 nM rhizoxin (Fig. 4B) reveals greatly reduced numbers of zoids and 1K2N cells. Also, in comparison to the 5 nM treatment there is only a small fall in the percentage of 1K1N cells when compared to controls. A closer examination of the production of zoids after rhizoxin treatment shows the phenomenon to be concentration dependent (Fig. 4C). High numbers of these cytoplasts are only produced in low rhizoxin concentrations, with a peak production around 5 nM (Fig. 4C).

**Fate of rhizoxin treated trypanosomes relative to their position in the cell cycle**

The cell cycle length for these procyclic parasites is 8.5 hours. Therefore, to define the relationships between periodic microtubule events and their sensitivity to rhizoxin during one cell cycle we studied the pattern of accumulation of aberrant cells during an 8 hour period after drug addition. Since we needed to know at which point individual trypanosomes were in their cell cycle when rhizoxin treatment began, we labelled the cell population with the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), a marker for DNA synthesis. A mid-log phase culture was incubated with BrdU for 1 hour to label cells that were in S phase during this hour. The cells were then washed in medium and the culture divided into a control and a 5 nM rhizoxin treated population. Samples were taken and stained with anti-BrdU antibody and DAPI to allow classification of all cell types. The results of this experiment are seen in Fig. 5. These results show that zoids are not produced immediately after the drug is added, suggesting that cells need to be exposed to the drug for some time before the result of the division produces a zoid. Since the main accumulation of zoids occurs after 4 hours (Fig. 5B), it is likely that cells which produce these zoids were in the early stages of their cell cycle when the drug was added. This means that they were probably in either G1 or S phase. This ability of cells late in their cell cycle to escape the effects of 5 nM rhizoxin in the first few hours of exposure is reflected in the fact that there is no significant drop in the numbers of 1K1N and 2K2N cells for up to 3 to 4 hours. If we consider the 1K2N cells (Fig. 5B), the number of this aberrant cell type increases around the same time as the zoids, suggesting that 1K2N cells are the siblings of the zoids. The nuclear configuration of these cells suggests that mitosis or post-mitotic nuclear segregation was compromised by rhizoxin but that cell division was able to proceed. This conclusion is also supported by the cytological evidence discussed earlier, where individual cells that appear to be in the process of division would produce a zoid (1K0N) and a 1K2N cell (Fig. 3E,E’).

The increase in zoid number parallels the rise in 1K2N cells; however, later on after treatment there are more zoids than 1K2N cells. This is seen clearly in the 8 hour count data (Fig. 4A) indicating that there must be another sibling cell type formed at a division with a zoid. The evidence suggests that these are cytologically 1K1N cells but that they have a 4C DNA content, the nuclear DNA having replicated but the cell failing to undergo mitosis. There are two lines of evidence to support this conclusion. Firstly, we have shown previously by quantification of nuclear DNA that the 1K1N population after rhizoxin treatment contains a sub-population of cells with elevated DNA contents (Robinson et al., 1995). Furthermore, the direct cytological examination of cell types in 5 nM rhizoxin reveals cells with a 2K1N configuration that are dividing to produce a zoid (1K0N) and a 1K1N cell (Fig. 3D,D’). Thus, importantly, in these cells even though mitosis has failed, the cells still go through a cell division. Note that a normal 1K1N cell cannot be easily distinguished from a 1K1N cell with an increased (>2C) nuclear content merely by non-quantitative cytological screening. To distinguish such cells with elevated nuclear DNA content, born as siblings of zoids, we refer to them.
as 1K1N*. In this context, we note that the 1K1N cell numbers start to fall as a percentage between 5-8 hours (Fig. 5A). This can be explained as a result of division of cells, 5-8 hours after the introduction of the rhizoxin, producing only one 1K1N cell (the 1K1N* cell) and a zoid. Therefore, only one 1K1N cell (the 1K1N* cell) is returned to the population rather than the two 1K1N cells of a normal control division.

Analysis of the BrdU data of 1K2N cells (Fig. 5B) shows that there are no ‘nucleus only’ BrdU labelled cells in this category. This suggests that cells which were in the window of the cell cycle between the end of SK and the end of SN when the rhizoxin was added were able to progress through mitosis and divide properly to two 1K1N siblings. Between 4 and 6 hours after drug exposure the increase in the K and N labelled 1K2N cells is very close to the pattern of the total 1K2N cell population. This suggests that most of these cells result from the division of cells that were in the SK / SN phase window of the cell cycle when rhizoxin was added. This is consistent with the fact that these cells were in the early part of the SK phase when the BrdU was removed from the medium, since SK starts slightly before SN (Fig. 1; Woodward and Gull, 1990).

This latter result suggests that there is a ‘break point’ around the end of SK when, even though 5 nM rhizoxin is now present, the cell is still able to go on and complete mitosis and cell cleavage. If the cell is at an earlier point in its cell cycle, in SK or the SK/SN overlap region, when it first encounters the rhizoxin, then it appears to go on to be compromised in mitosis but to divide to give a 1K2N cell and a zoid. There is also a rise in unlabelled 1K2N cells late, at around 7 hours (Fig. 5B), and these could represent cells that were again earlier than the start of SK when the drug was added. These numbers do not rise dramatically, suggesting that the switch to another type of sibling in a zoid producing division is somewhere in late G1.

The increase in numbers of labelled zoids closely follows the total zoid increase with most of the zoids produced at around 4 to 6 hours being labelled (Fig. 5B). This supports the conclusion that the zoids are being produced from cells that were in the SK phase or the SK/SN overlap region when the drug was added and mirrors the conclusions from the analysis of the above 1K2N cell data. These 1K2N cells are of course the siblings of these early zoids. At a slightly later time there is an increasing appearance of unlabelled zoids (increasing in numbers after around 6 hours). It is possible that these again originate from cells that were in G1 phase when the drug was added. These could have been the siblings of either the unlabelled 1K2N cells or unlabelled 1K1N* cells (the latter are again cytologically impossible to differentiate from normal 1K1N cells in routine DAPI staining). This analysis of the effects of a low concentration of rhizoxin (5 nM) on the microtubule mediated events of the cell cycle reveals markedly differing outcomes for individual cells at particular points in the cell cycle.

Fig. 5. Graphic representation of T. brucei cell types, during 8 hours rhizoxin treatment (5 nM), following 1 hour labelling with BrdU. The percentage of cell types with each particular kinetoplast (K) and nuclear (N) configuration is shown (as determined by DAPI staining of the kinetoplast and nucleus). In (A) note the decrease of the 1K1N cells in the treated population after 5 hours in rhizoxin. In (B) the total percentage of aberrant cell types occurring in the rhizoxin-treated population is shown (zoids and 1K2N cells); this includes the BrdU labelling of the nucleus only, of the kinetoplast only, or both. The BrdU labelled cells are the proportion of the rhizoxin treated culture which were at either SK or SN or both SK/SN during the 1 hour incubation with BrdU.
**Inhibition of nuclear S phase ($S_N$) by aphidicolin**

Given that rhizoxin treated cells that fail mitosis still divide (to form a zoid and a particular cell type), we then questioned the effect of inhibition of nuclear DNA synthesis with aphidicolin.

60 μM aphidicolin effectively inhibits incorporation of BrdU into nuclear DNA but not into the kinetoplast DNA (data not shown). This is in agreement with previous studies with other trypanosomes showing the specificity of aphidicolin as an inhibitor of DNA polymerase α and therefore of nuclear specific DNA synthesis (Zimmerman et al., 1980; Rojas et al., 1993; Itoh et al., 1996). Also, FACS analysis of aphidicolin arrested *T. brucei* cells indicates that after 10 hours most trypanosomes are held in nuclear S phase (Fig. 6B).

10 hour treatment with 60 μM aphidicolin resulted in a reduction of post-mitotic cells (2K2N) from approximately 7% (found in control cultures) to 0% after approximately 6 hours (Fig. 7A). This suggests that trypanosomes with inhibited nuclear DNA synthesis do not subsequently enter mitosis and also that the ‘mitotic entry checkpoint’ found in most eukaryotes appears to be functioning in this organism.

Concomitant with the reduction of post-mitotic cells from the population is a rise in the number of zoids (Fig. 7B). Quantification of the types within the cell population after 10 hour treatment with aphidicolin (Fig. 7C) shows interesting comparisons with the 5 nM rhizoxin experiment (Fig. 4A). Both drugs produce zoids, but in contrast to the 5 nM rhizoxin treatment there is an almost equivalent reduction in the number of 1K1N cells after aphidicolin treatment to the number of zoids produced. This, taken with the rise in the number of 2K1N cells, is highly indicative of the fact that these 2K1N cells are committed to divide. Despite of the aphidicolin inhibition of nuclear S phase, these cells progress through the cell cycle, do not enter mitosis, but do divide as 2K1N cells into a zoid (1K0N) and a 1K1N cell.

FACS analysis of a trypanosome culture treated for 10 hours with aphidicolin reveals a few cells with a peak of fluorescence smaller than the peak of the $S_N$-arrested cells (Fig. 6B). A scatter plot of this culture reveals that the cells in the smaller peak are much smaller in size than the majority of cells in the population, indicating that these cells represent the zoid fraction (Fig. 6D).

**Disruption of a nuclear-mitochondrial checkpoint?**

Our DAPI and FACS analysis of the aphidicolin-treated trypanosomes showed that inhibition of $S_N$ in *T. brucei* results in inhibition of mitosis but not inhibition of cytokinesis. Such aberrant division produces a 1K1N cell and an anucleate zoid (1K0N). However, further insight comes from using the monoclonal antibodies BBA4 and ROD1, which label the basal body and the paraflagellar rod, respectively (Woods et al., 1989), to investigate this 1KIN population. Aphidicolin treated trypanosomes produced a population of cells with a single kinetoplast and nucleus (1KIN), seemingly G1 cells by DAPI staining (Fig. 8). In fact, this (1KIN) population consists of two distinctly different sub-populations. Cells with a single basal body (as anticipated for G1 trypanosomes; Fig. 8A) comprise the majority of this cell type (approximately 90% of the 1KIN population). Interestingly, however, there are also cells with two basal bodies, as anticipated for G2 trypanosomes (Fig. 8B,C). In the latter case, the single kinetoplast is associated either with the anterior/old basal body (Fig. 8B',B'') or with the posterior/new basal body (Fig. 8C). These are G1 cells in terms of their DAPI stained DNA configuration, but G2 in terms of their basal body content and position. In addition, the staining with the anti-PFR antibody ROD1 reveals that these cells have a single paraflagellar rod, as G1 cells would be expected to have. Therefore, the minority of cells in this 1KIN population diverge from the normal cell cycle, in that they possess both G1 and G2 characteristics, suggesting a disruption between nuclear and kinetoplast/basal body/flagellum, cell cycle co-ordination.

**DISCUSSION**

We have shown previously that maytansine and ansamitocin inhibit microtubule-mediated processes in *T. brucei* (Robinson and Gull, 1991). These compounds and rhizoxin have been shown to bind to tubulin at the ‘vinblastine site’ (Mandelbaum-
This concentration of drug allows cytokinesis and the microtubule-mediated process most affected is mitosis. Mitosis in *T. brucei* is intranuclear and initially the chromosomes are segregated by the mitotic spindle. Subsequently, the microtubule spindle elongates, separating the two daughter nuclei to specific cellular locations (Sherwin and Gull, 1989a; Robinson et al., 1995; Ersfeld and Gull, 1997). Thus, inhibition of the first event (spindle formation) would be likely to lead to complete inhibition of mitosis. If cytokinesis ensued then it would produce a zoid plus a cell with a single nucleus (Fig. 9). This nucleus (N*) would possess a replicated but unsegregated genome (Robinson et al., 1995). Alternatively, rhizoxin inhibition of only the second mitotic event (spindle elongation/nuclear positioning function) may lead to incomplete mitosis (Fig. 9). Interestingly, the *tub2-401* β-tubulin mutant, of *S. cerevisiae* (Sullivan and Huffaker, 1992), goes through the first mitotic event but fails to position the daughter nuclei correctly; the ensuing division in this case also produces a binucleate and an anucleate cell.

The outcome of a particular cell division is dependent upon the position of the cell in the cell cycle when the drug was first added. An overview of these conclusions is shown in the diagram in Fig. 9B. We suggest there are three major windows in the cell cycle wherein a cell will exhibit a different fate at the forthcoming division at the end of its cell cycle. Cells late in their cell cycles when drug is added are essentially unaffected and complete both mitosis and cell division. At 5 nM rhizoxin this window of the cell cycle is defined as from the end of *S* to the end of the cell cycle (between 0.5 and 1.0 of the unit cell cycle; Woodward and Gull, 1990). Cells earlier in the cell cycle define a second window encompassing late *G*1, the initial early *S* period and the overlap period of the rest of *S* with the early part of *G*2. Cells in this window of the cell cycle when rhizoxin is added are subsequently

Mitosis and cytokinesis

The use of rhizoxin at 5 nM concentration is more illuminating than the 100 nM experiments.

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**Fig. 7.** *T. brucei* cell types in a population treated with 60 μM aphidicolin. (A) Post-mitotic (2K2N) *T. brucei* cells of control and aphidicolin treated cultures. During a 10 hour aphidicolin treatment the reduction in the 2K2N cells begins as early as 2 hours. (B) Zoid formation occurs within 4 hours of drug treatment and it coincides with the reduction of post-mitotic cells in the aphidicolin-treated population. (C) Cell types of *T. brucei* control and aphidicolin treated (10 hours) culture. The cell types are classified in terms of kinetoplast and nuclear cell content. Note the reduction in post-mitotic cells coupled with increase of the 2K1N cell type, in addition to the reduction of 1K1N cells in this treated population.

**Fig. 8.** *T. brucei* 1K1N cell types in a population treated with 60 μM aphidicolin. In (A’, B’, C’) DAPI fluorescence shows that the cells possess a single kinetoplast and a single nucleus (1K1N cells). (A”, B”, C”) Double immunofluorescence with the antibodies BBA4 (labels the basal body) and ROD1 (labels the paraflagellar rod) reveals that the 1K1N cell population consists in fact of three different cell types. Cells with a single flagellum and basal body (A”) and cells with a single flagellum but two basal bodies, of which either the anterior (B”) or the posterior (C”) can be associated with the single kinetoplast. Arrows indicate the kinetoplast and its associated basal body, (in the trypanosomes possessing two basal bodies). Bar, 3.5 μm.
compromised in either mitosis or post-mitotic nuclear positioning, yet still proceed on to cleave at cytokinesis. This cleavage produces a zoid (1K0N) and a 1K2N cell. The third window of the cell cycle that is defined by treatment with 5 nM rhizoxin encompasses most of the G1 period. Cells, that are in this window at the time of rhizoxin addition are compromised in mitosis, and little or no nuclear segregation occurs. Yet, even though mitosis has failed the cell continues to progress through cytokinesis. This division produces a zoid (1K0N) and a 1K1N* cell. (The 1K1N* designation denotes the fact that this cell has a different nuclear DNA content to a normal control 1K1N daughter cell.)

The microtubule mediated process most sensitive to low rhizoxin concentrations seems to be mitosis, including both the chromosome segregation and the later nuclear positioning events. In most eukaryotic cells an important overall cell cycle checkpoint ensures that a failure of mitosis leads to a failure of cytokinesis, so ensuring that the cell does not divide if mitosis has not yet been completed. The study of cut mutants in S. pombe has shown the importance of such a control mechanism between mitosis and cytokinesis. Abolition of this control allows cytokinesis to proceed independently of defects in nuclear division, with lethal effects; cells are produced in which an undivided nucleus is positioned in the middle of the septum plane (Hirano et al., 1986; Yanagida, 1998).

Earlier reports of the effect of high concentrations of other less effective antimicrotubule agents have noted the production of anucleate cytoplasts, although their mechanism of production and significance was not determined, (Ono and Nakabayashi, 1979). The other phenomenon, revealed by the use of low rhizoxin concentrations on T. brucei, is that those trypanosomes with compromised mitosis which continue on and divide (forming a 1K0N zoid) have completed the D period of their cell cycle (kinetoplast segregation). Normal cytokinesis in T. brucei involves a longitudinal cleavage of the cortical cytoskeleton that bisects the cell between the new and old flagellum and the segregated kinetoplasts and nuclei. In 5 nM rhizoxin the division occurs to cleave off a posterior portion of the cell with the new flagellum and associated basal body, a kinetoplast and mitochondrion. Since there is no daughter nucleus positioned in this portion of the cell, the result is the formation of a zoid (1K0N) as one of the siblings. The T. brucei cell may well possess novel cell cycle checkpoint controls, in that entry into cytokinesis is linked more to completion of the D/early A phase and basal body segregation than to mitosis.

These experiments show that the various microtubule mediated processes of the trypanosome cell can be selectively inhibited by different concentrations of the powerful antimicrotubule agent rhizoxin. A detailed analysis of the cytokinetic events that result in zoid production shows that although mitosis is inhibited, T. brucei cells still go on to perform cytokinesis, suggesting that the usual mitosis to cytokinesis checkpoint does not operate in trypanosomes. Given such a situation, one model could be that there may exist checkpoints which operate to link completion of both S phases (SK and SN) to subsequent segregation of these two unit genomes by kinetoplast segregation (D/early A phase) and mitosis (M phase). Moreover, since segregation of the mitochondrial genome (D/early A phase) is accomplished before mitosis is initiated it may be that either or both the late events of mitosis and cytokinesis in trypanosomes show cell cycle checkpoint links to this earlier and clearly important cell cycle event of kinetoplast segregation.

![Fig. 9. Summary of the T. brucei kinetoplast and nucleus cell cycle, subdivided into three periods. These periods are defined by the fate of cells when treated by a low concentration (5 nM) of rhizoxin. Cells late in their cell cycle (end of SN, kinetoplast G2 or later) at the time of rhizoxin addition, seem to proceed unaffected within the cell cycle (Ai,B). These cells complete the subsequent phases correctly, dividing finally to two 1K1N cells. Cells in late G1, S or S/early S proceed in their cell cycle but may suffer compromised mitosis and post-mitotic positioning of the two nuclei (Aii,B). This results in aberrant cytokinesis forming a 1K2N cell and a zoid. G1 cells are the most affected by rhizoxin treatment: they proceed through S phase and replicate their genomes but fail mitosis (Aiii,B). The subsequent division produces a zoid and a 1K1N* cell with a replicated but unsegregated nuclear genome.](image)
Nuclear DNA synthesis (SN) and cytokinesis
Since inhibition of mitosis did not block cytokinesis, we considered whether inhibition of nuclear DNA synthesis would have an effect (1) on mitosis and (2) on cytokinesis. The selectivity of aphidicolin (used at concentration of 60 μM) for nuclear DNA synthesis (SN) inhibits kinetoplast DNA synthesis (SK) to proceed. The aphidicolin experiment revealed two phenomena. First, as in most eukaryotes, in T. brucei inhibition of SN is followed by inhibition of mitosis, revealing that the mitotic entry checkpoint is functioning in trypanosomes. Second, contrary to what is observed for most eukaryotic cells in trypanosomes after mitosis inhibition the cell cycle proceeds, cytokinesis occurs and anucleate cells are formed. In agreement with our result, Ono and Nakabayashi (1980) observed that treatment of T. gambiense with bleomycin (which inhibits SN but not SK) resulted in the decrease of 2K2N cells, increase in 2K1N cells and production of 1K0N (anucleate) cells. Such a cytokinesis event suggests the lack of a cell cycle checkpoint preventing the trypanosome from undergoing cytokinesis in the absence of mitosis or nuclear DNA synthesis.

Novel cell cycle controls in Trypanosoma brucei?
Our rhizoxin and aphidicolin experiments show that in T. brucei inhibition of mitosis or nuclear DNA synthesis does not cause inhibition of cytokinesis. This suggests that T. brucei lacks checkpoints which would prevent the trypanosome cell from undergoing cytokinesis in the absence of mitosis or nuclear DNA synthesis and secondly, that the T. brucei cell cycle checkpoints are focused on cellular events other than mitosis or nuclear DNA synthesis.

This raises the issue of how are the cell cycle controls of this organism operating. In embryonic cells a timing cell cycle control mechanism has been observed, to prevent initiation of a cell cycle event before completion of a previous one (Hara et al., 1980; Raff and Glover, 1988). An event in the cell cycle is completed in good time before the next occurs, therefore the correct procession of cell cycle events depends on their timing. Such a control mechanism could partly explain our results. A normal rate of spindle formation/assembly would place the daughter nuclei in the correct position, before cell division. If rhizoxin delayed the formation of the spindle, then the cell would not place the nuclei in the correct position at the correct time prior to cell division (with consequent formation of a 1K2N cell and a zoid).

Trypanosomes need to replicate and segregate two single unit genomes, in addition to the duplication and segregation of other cell structures/organelles. Our studies of the cytokinetic process in T. brucei point towards the presence of a novel cell cycle checkpoint regulating trypanosome cell division. The common parameters in both the rhizoxin experiments (M inhibition) and the aphidicolin experiments (SN inhibition and subsequent M failure) are (1) that cytokinesis occurs and (2) that the SK, D and A phases of the kinetoplast are essentially unaffected. The question therefore arises, could control over cell cytokinesis be exercised by one of the kinetoplast cell cycle phases, synthesis, division or segregation? Experiments with hydroxyristabolise, p-rosaniline and furazolidon, which affect the kinetoplast, have been reported to induce the formation of akinetoplast trypanosomes (Ono and Inoki, 1975, 1976). Although these compounds allow cell division, it is unclear exactly how they interact with the kinetoplast and thus it is difficult to draw exact conclusions from these experiments. Treatment with okadaic acid, a protein phosphatase inhibitor at a range of concentrations, inhibits cytokinesis in T. brucei (Das et al., 1994), but neither SK nor SN was inhibited (demonstrated by BrdU labelling) and the presence of multiple nuclei suggested that mitosis was not inhibited. In both cases the authors did not investigate the relationship between the flagellum basal bodies and the kinetoplast and so did not report on the presence, absence or importance of basal bodies in the drug treated cells. The presence of a single kinetoplast in okadaic acid treated cells indicates that the D period of the kinetoplast was compromised, suggesting a possible regulatory relationship between kinetoplast segregation and cytokinesis. Given such a situation, one model could be that there may exist checkpoints which operate to link completion of both S phases (SK and SN) to subsequent segregation of these two genomes by basal body segregation and mitosis. Moreover, since segregation of the mitochondrial genome (D/early A phase) is accomplished before mitosis is initiated, it may be that either or both the late events of mitosis and cytokinesis in trypanosomes show cell cycle checkpoint links to kinetoplast and/or basal body function or segregation.

Further studies are required to elucidate the role of kinetoplast segregation upon cell cytokinesis in trypanosomes. Our aphidicolin experiments suggest that such studies will need to consider the cell’s basal bodies. We have shown previously that kinetoplast segregation is basal body mediated (Robinson and Gull, 1991). Our use of monoclonal antibodies as probes for cytoplasmic events has shown that the DAPI images of drug treated cells do not always reflect their true cell cycle position. Some apparently 1K1N cells are really in G2, as judged by their cytoplasmic basal body configuration. Importantly, however, in these cells there appears to be no formation of new flagellum (as judged by the absence of a PFR) on the new basal body complex. Basal body replication is initiated at the start of nuclear S phase (Woodward and Gull, 1990) and axoneme/PFR formation only occurs subsequently. Thus this drug induced phenotype may reflect one form of checkpoint control operating between completion of nuclear DNA synthesis and activation of cytoplasmic events associated with subsequent cytokinesis.

Likely cell cycle regulatory molecules are being identified in kinetoplastid protzoa (Doerig et al., 1999). However, our results indicate that the trypanosome cell may possess novel cell cycle control features. Kinetoplastid protozoa possess a periodic S phase for both nuclear and mitochondrial DNA and need to ensure fidelity of segregation of the mitochondrial unit genome as a periodic event prior to mitosis and cytokinesis. Future reverse genetic analysis of these regulatory molecules will prove illuminating for this important area of parasite biology.

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