

## Cell cycle-specific induction of an 89 kDa serine/threonine protein kinase activity in *Trypanosoma brucei*

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### SUMMARY

The cell cycle compartmentalization of specific activities of the protozoan parasite *Trypanosoma brucei* has remained unexplored due to the lack of a cell synchronization protocol. We report here that stationary phase cells stimulated to enter the cell cycle showed significant synchrony through the first cycle. The pattern of tyrosine phosphorylated proteins, known to undergo alterations during trypanosome development, showed only moderate changes as quiescent cells entered the cycle, particularly an increase in a 77 kDa species. However, the activity of an 89 kDa protein kinase (SPK89), previously demonstrated to be restricted to the proliferative stages of the parasite's life cycle, markedly increased as the population entered S phase. Cell sorting experiments demonstrated that SPK89 activity was

highest in S phase cells and moderate in G<sub>2</sub>/M cells. The entry into S phase and increased SPK89 activity did not depend on serum factors but required protein synthesis for a discrete period after stimulation. Various modulators of protein phosphorylation were tested to determine their effects on progression to S and SPK89 activity. Only staurosporine and genistein were effective. However, both of these compounds inhibited virtually all protein phosphorylation and protein synthesis in the parasites. Thus these drugs cannot be used as specific protein kinase inhibitors in trypanosomes.

Key words: cell cycle, protein kinase, protein phosphorylation, trypanosome, *Trypanosoma brucei*, tyrosine phosphorylation

### INTRODUCTION

Proliferation of eukaryotic cells is regulated in part by differential protein phosphorylation within the cell cycle (Nasmyth, 1993; Pouyssegur and Seuwen, 1992; Pelech and Sanghera, 1992). Thus, protein kinases, the enzymatic mediators of protein phosphorylation, play key roles in cell cycle progression. Indeed, several cytoplasmic protein kinase activities appear to be cell cycle regulated. The most well studied of these are members of the p34<sup>cdc2</sup> gene family, which have been found in all eukaryotes examined thus far (Draetta, 1990). The cell cycle-specific activity of the p34<sup>cdc2</sup> protein kinase leads to induction of DNA synthesis (S phase) (Roberts, 1993) or onset of mitosis (M phase) (Norbury and Nurse, 1992; Nurse, 1990). While regulation of the cell cycle by p34<sup>cdc2</sup> appears to be a common control mechanism of eukaryotic cell proliferation, differential activity of other protein kinases within the cell cycle such as Spk1 (Zheng et al., 1993), pp60<sup>c-src</sup> (Bagrodia et al., 1991), PTK72 (Burg et al., 1993), Cdc7 (Jackson et al., 1993; Yoon et al., 1993) and NIMA (Osmani et al., 1991) suggests that regulation of cell growth is multi-component, and includes an interdependent phosphorylation/dephosphorylation network. In addition, in many systems entry into G<sub>1</sub> from a previous quiescent (G<sub>0</sub>) state is accompanied by dramatic increases in tyrosine phosphorylation of specific

proteins (see, for example, Cooper et al., 1982; June et al., 1990; Taverna and Langdon, 1973; Herbst et al., 1991; Weinstein et al., 1992).

While much information has been gathered on key processes required for cell cycle progression in metazoans and yeasts, virtually no information exists about the signals involved in cell cycle control in more ancient eukaryotes. The evolutionary distance between the protozoan parasite *Trypanosoma brucei* and humans is about twice that between humans and yeast (Sogin et al., 1989). This parasite and related species are the causative agents of African sleeping sickness in humans and a similar disease in cattle, placing over 50 million people at risk and rendering a large part of the African continent unsuitable for agricultural development (World Health Organization, 1986). The complex life cycle of African trypanosomes involves alternation between proliferative and quiescent stages in both mammalian and insect hosts (Vickerman, 1985). Infective parasites are transmitted to the mammalian host through the bite of the tsetse fly vector and quickly establish themselves as proliferative slender bloodforms. After several rounds of proliferation, slender bloodforms eventually differentiate into quiescent stumpy bloodforms, which are ingested by the feeding insect vector. Several developmental changes occur within the insect, including differentiation to proliferative procyclic form parasites, leading to eventual cyclical transmission.

The unusual genetic processes of trypanosomes, such as antigenic variation (Van der Ploeg et al., 1992), trans-splicing (Tschudi and Ullu, 1990; Huang and Van der Ploeg, 1991) and RNA editing (Hajduk et al., 1993) have attracted numerous molecular biologists to *T. brucei*, making it perhaps the most-well studied parasite at the genetic and molecular levels. In contrast, little is known about the role of protein phosphorylation in the control of proliferation and differentiation of these organisms. Previous studies have determined that specific proteins are phosphorylated on serine, threonine and tyrosine residues at definite stages within the life cycle (Aboagye-Kwarteng et al., 1991; Parsons et al., 1991), indicating that serine/threonine and tyrosine protein kinases and/or their substrates are regulated in a developmental fashion. Recently, we have described several electrophoretically distinct protein kinase activities in *T. brucei*, many of which appear to be developmentally regulated (Parsons et al., 1993). Among these is an 89 kDa serine/threonine protein kinase that exhibits highest activity in the proliferative slender bloodform (mammalian stage) and procyclic form (insect stage) developmental stages. Activity is minimal in non-dividing stumpy bloodforms and stationary phase procyclic forms (Parsons et al., 1993).

The present study was undertaken to assess the relationship between the activity of the 89 kDa protein kinase activity and the trypanosome cell cycle. We now name this protein kinase SPK89 for S phase protein kinase of 89 kDa. The cell-cycle-specific compartmentalization and kinetics of induction of SPK89 activity as resting cells enter the cell cycle suggests involvement of SPK89 in regulation of the trypanosome cell cycle.

## MATERIALS AND METHODS

### Parasites

Procyclic form organisms of the pleiomorphic TREU667 strain of *T. brucei* (Michelotti and Hajduk, 1987) were propagated in vitro in SDM-79 (Brun and Schonberger, 1979) plus 10% fetal bovine serum (SeruMax, Sigma). Procyclic forms were cultivated and harvested during logarithmic growth phase. Stationary phase procyclic form cultures were prepared by inoculating 30 ml cultures at  $10^7$  parasites/ml, in 75 cm<sup>2</sup> polystyrene tissue culture flasks. Culture flasks were incubated with gentle agitation with caps firmly tightened. With no subsequent feeding, the parasites proliferated for two days and then on day three were either harvested or stimulated to divide by dilution into fresh medium to a final concentration of  $10^7$ /ml. By this method semi-synchronous cultures were obtained and harvested at various time points after stimulation with fresh media. We have found that maintaining standard conditions regarding cell density and geometry of the cultures is important for the reproducibility of the procedure. For inhibitor studies, stationary phase procyclic form cultures were diluted into fresh media containing various inhibitors: cycloheximide (Sigma), tyrphostin A25 (Gibco BRL), genistein (Gibco BRL), H7 (Sigma), H8 (Calbiochem), staurosporine (Calbiochem) or okadaic acid (Gibco BRL). Cells were harvested and lysed in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, heated in a boiling bath for 5 minutes, and stored at  $-80^{\circ}\text{C}$  until use.

### Metabolic labeling

Stationary phase cells were stimulated as detailed above except that methionine-free medium containing 10% dialyzed serum and 200  $\mu\text{Ci/ml}$  of [<sup>35</sup>S]methionine was used in place of complete medium. Parallel cultures were incubated in the presence or absence of various inhibitors and harvested at 5 hours as above. Proteins were precipi-

tated with 10% trichloroacetic acid (TCA) and [<sup>35</sup>S]methionine incorporation was quantitated by scintillation counting. For phosphate labeling, exponential procyclic form cultures were incubated for 16 hours with various inhibitors in the presence of [<sup>32</sup>P]orthophosphoric acid essentially as described (Parsons et al., 1991). Labeled cells were washed and harvested as for SDS-PAGE.

### Protein kinase renaturation assay

A total of  $10^7$  cells were loaded per lane for analysis on 10% SDS-PAGE (Laemmli, 1970). For sorted populations of cells (see below),  $4 \times 10^6$  methanol-fixed cell equivalents were loaded per lane. Electrophoresed proteins were transferred to polyvinylidenedifluoride membranes (Millipore) and subjected to the kinase renaturation assay as previously described (Parsons et al., 1993), using a modification of the procedure originally described by Ferrell and Martin (1989). Briefly, bound proteins were denatured by incubating the blot in 7 M guanidine-HCl for 1 hour, after which the blot was rinsed and placed in renaturation buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1% bovine serum albumin, 0.1% Nonidet P40, pH 7.5) for an overnight incubation at  $4^{\circ}\text{C}$ . After renaturation the blots were blocked, washed and incubated for 1 hour in labeling buffer (30 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>) in the presence of 50  $\mu\text{Ci/ml}$  [ $\gamma$ -<sup>32</sup>P]ATP. Following several washes, the blots were dried and placed on film for autoradiography. By this method the locations of the various electrophoretically distinct, enzymatically active protein kinases can be revealed by autoradiography. In some experiments the membranes were incubated in labeling buffer in the presence of various protein kinase inhibitors. Phosphorimager analysis of each blot was performed as described (Parsons et al., 1993).

### Anti-phosphotyrosine immunoblot analysis

Affinity-purified anti-phosphotyrosine antibodies were a gift from Drs Jeffrey Ledbetter and Gary Schieven (Bristol-Myers/Squibb Pharmaceutical Research Institute, Seattle, WA). Tyrosine-phosphorylated proteins in trypanosome cell lysates ( $10^7$  cells/lane) were detected on immunoblots using methodologies previously described (Parsons et al., 1991).

### Flow cytometric analysis and cell sorting

For analysis of DNA content, cells were harvested by centrifugation, washed once with phosphate buffered saline, pH 7.2 (PBS), and fixed at room temperature for 1 hour in 70% methanol/PBS at a concentration of  $10^7$ /ml. Following fixation, cells were washed once in PBS, resuspended at  $10^7$ /ml in PBS and incubated for 20 minutes at  $37^{\circ}\text{C}$  in the presence of 10  $\mu\text{g/ml}$  RNase A. Propidium iodide (PI; Sigma) was added to the fixed cells at a final concentration of 10  $\mu\text{g/ml}$  and incubated for 1 hour at  $37^{\circ}\text{C}$ . For cell cycle analysis, 10,000 cells were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) or an Ortho Cyto Fluorograph equipped with a 585/42 band pass filter for the quantitation of red fluorescence intensity. Light-scatter gates were set to exclude dead cells, cell fragments and cell clumps from the analysis. Additionally, cells were gated on height versus width of the red fluorescence signal so as to exclude cell doublets from the analysis. For cell sorting, PI-stained cells were prepared from exponentially growing cultures and sorted using a FACStar Plus flow cytometer (Becton Dickinson Immunocytometry Systems) to obtain G<sub>1</sub>, S and G<sub>2</sub>/M populations. The sorted populations were recovered by centrifugation in bovine serum albumin-coated tubes, rinsed once, counted and prepared for SDS-PAGE.

## RESULTS

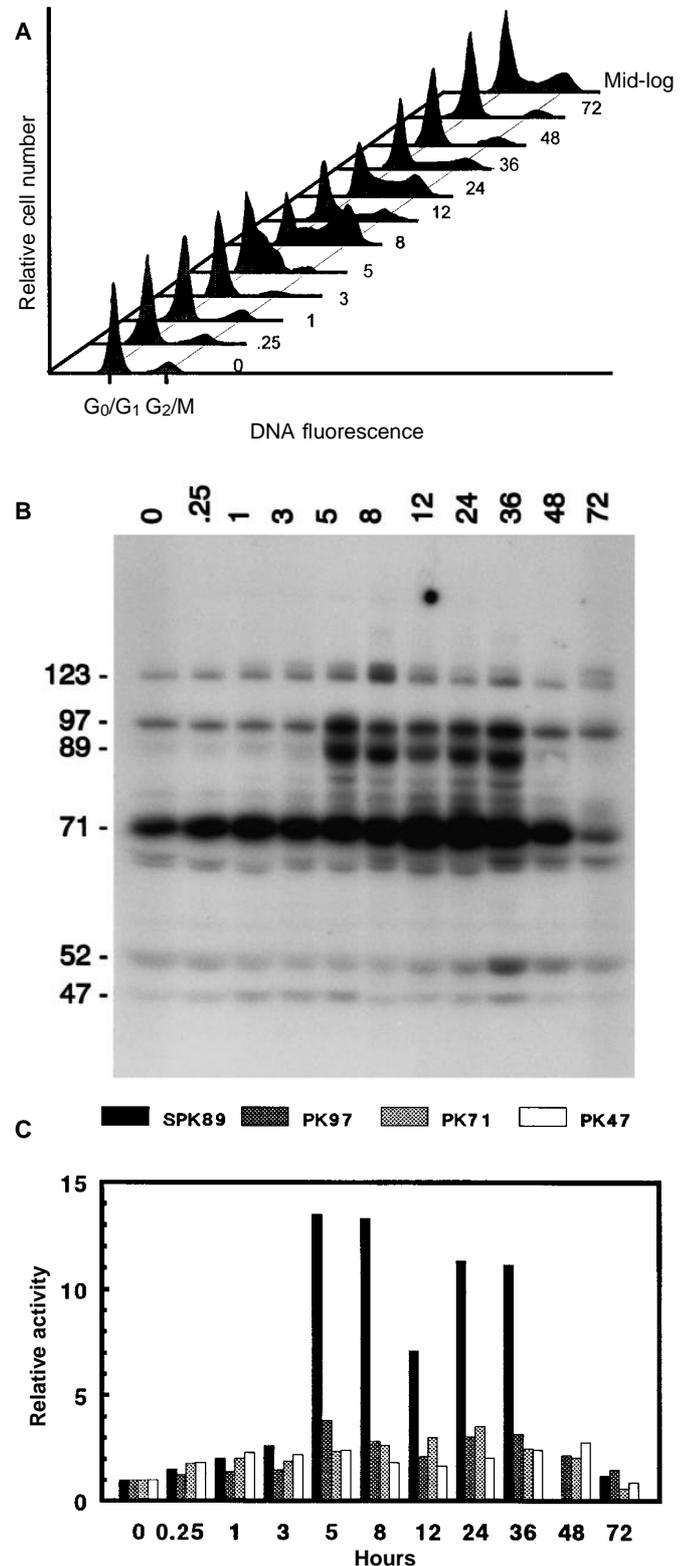
### Cell cycle analysis of semi-synchronous cultures

Previously, we identified an 89 kDa serine/threonine protein kinase activity (SPK89) which is restricted to proliferating parasites of both bloodform (which parasitize the mammalian

host) and procyclic form (which parasitize the tsetse fly midgut) stages (Parsons et al., 1993). These studies suggested that SPK89 activity may be regulated within the trypanosome cell cycle. Thus, we were interested in assessing the kinetics of induction of SPK89 activity as resting cells enter and progress through the cell cycle. No methodologies currently exist for obtaining synchronous populations of *T. brucei*. A moderate degree of synchrony has been obtained by nutrient deprivation of culture-adapted bloodforms (Morgan et al., 1993), but with a low yield. Our initial studies therefore involved establishing a culture method for increasing the synchrony of procyclic form trypanosomes in vitro. We prepared stationary phase procyclic form cultures and harvested them at various time points after stimulation with fresh media. Samples of cells were then subjected to flow cytometric analysis of DNA content (revealed by staining with PI) and kinase renaturation analysis.

Fig. 1A shows the fluorescence histograms obtained from flow cytometric analysis of the PI-stained cells. Like previous analyses of trypanosome populations (Kooy et al., 1989; Morgan et al., 1993), the histogram generated from an exponentially dividing procyclic form culture (Fig. 1A, upper histogram) resembles a classical cell cycle curve for exponentially dividing cells, having a major G<sub>0</sub>/G<sub>1</sub> population (2C chromosome number), with small S phase and G<sub>2</sub>/M populations (active DNA synthesis and 4C chromosome number, respectively). Cells harvested from the stationary phase culture were predominantly in G<sub>0</sub>/G<sub>1</sub>, with few cells in G<sub>2</sub>/M and no S phase. Microscopic analysis indicated that the G<sub>2</sub>/M population seen by flow cytometry contained approximately 40% post-mitotic cells (data not shown). The DNA profile was consistent through 3 hours post-stimulation. At 5 hours post-stimulation a large S phase population was observed, followed by a shift to G<sub>2</sub>/M at the 8 hour time point, indicating that the parasites were progressing through the cell cycle in semi-synchronous fashion. Simultaneous cell counting (data not shown) revealed that by 12 hours the parasites had undergone one round of cell division, similar to previous reports on the timing of the *T. brucei* cell cycle (Woodward and Gull, 1990). From

12 to 36 hours post-stimulation the parasites were rapidly proliferating, and the corresponding DNA histograms show rapid loss of synchrony. Stationary phase was reached by 48-72 hours. Thus, under the stated culture conditions, it is possible to obtain *T. brucei* procyclic form cultures that maintain partial synchrony through one round of cell division. Future studies



**Fig. 1.** Analysis of DNA content and SPK89 activity in semi-synchronous procyclic form cultures. Stationary phase cultures were prepared as described, stimulated at time 0, and harvested at the indicated time points thereafter. All analyses are from the same experiment. (A) Flow cytometric analysis of cellular DNA content. Cells were harvested at the time points indicated in hours (right of each histogram), stained with PI, and analyzed by flow cytometry. Each histogram represents data collected from 10,000 cells and is plotted on a linear scale. The positions of the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M peaks are indicated on the x axis. The top histogram represents the DNA profile obtained from cells harvested from a mid-log control culture. (B) Protein kinase renaturation analysis autoradiogram. Total cell lysates were prepared at the indicated time points (at top, in hours) and subjected to the kinase renaturation assay. Molecular masses (in kDa) of the various protein kinase activities are indicated at left, as determined by their migration on SDS-PAGE versus molecular mass standards. (C) Quantitation of protein kinase activities. Phosphorimager analysis of blot from protein kinase renaturation assay shown in Fig. 1B. For each protein kinase, relative activity is shown in arbitrary units per µg protein, with the activity at 0 hours (stationary phase) defined as 1.0 unit. The activity of SPK89 at 48 hours was not quantitated due a bubble in that region of the blot.

requiring semi-synchronous trypanosome cell populations free of inhibitory chemicals may find this methodology useful.

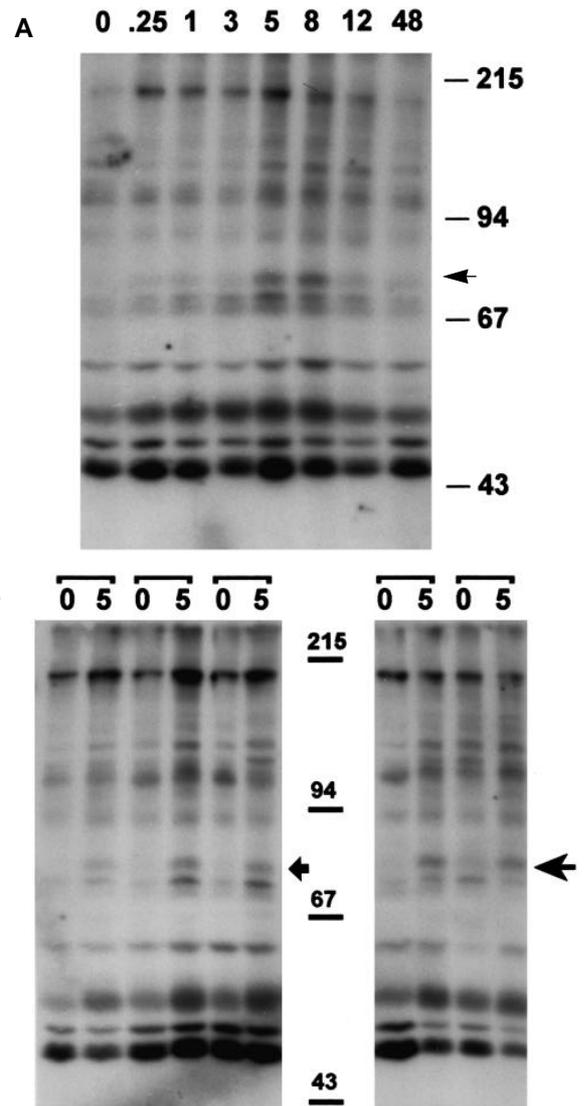
### Protein kinase renaturation analysis of semi-synchronous procyclic form cultures

Using cells harvested from the semi-synchronous cultures described above, we analyzed protein kinase activities by kinase renaturation assay. As seen in Fig. 1B, the stationary phase cells (time 0) possessed several electrophoretically distinct renaturable protein kinase activities, yet exhibited only minimal SPK89 activity, consistent with our previous results (Parsons et al., 1993). Little SPK89 activity was observed through 3 hours post-stimulation. A large increase in activity was observed at the 5 hour time point and continuing through 36 hours post-stimulation, corresponding to the duration of observed exponential cell growth. When the cultures had again reached stationary growth phase at 48-72 hours, SPK89 activity was reduced to very low levels.

Previously, we demonstrated that a given protein kinase activity observed in the kinase renaturation assay can be quantitated in a linear dose-response fashion by phosphorimaging (Parsons et al., 1993). We therefore performed phosphorimager analysis on selected radiolabeled bands (97 kDa, 89 kDa, 71 kDa and 47 kDa) seen on the kinase renaturation blot shown in Fig. 1B, arbitrarily defining the initial activities at time 0 as 1.0. As seen in Fig. 1C, while the activities of the other renaturable protein kinases were only moderately increased at 5 hours post-stimulation, the activity of SPK89 increased approximately 13-fold and remained relatively high throughout the ensuing cell growth. By 72 hours all renaturable protein kinase activity reached stationary phase levels. Thus, there was a strong and specific induction of SPK89 activity at the same time when a shift to S phase occurred in the semi-synchronous cultures.

### Tyrosine phosphorylation in semi-synchronized cells

When quiescent mammalian cells, such as serum-starved cells, are stimulated to enter the cell cycle, dramatic increases in the tyrosine phosphorylation of specific proteins are observed. To determine if intracellular protein tyrosine phosphorylation is induced as quiescent trypanosomes enter and progress through the cell cycle, we used anti-phosphotyrosine immunoblot analysis to examine the cell lysates prepared above. As seen in Fig. 2A, a moderate increase in the overall abundance of tyrosine-phosphorylated proteins was observed as the quiescent cells entered and progressed through the cell cycle, including a notable increase in the abundance of a 55 kDa species. A 77 kDa tyrosine-phosphorylated protein not seen in starved cells was observed in stimulated cells, especially at 5-8 hours post-stimulation. Analysis of five paired populations of starved and stimulated (5 hour) cells showed that this induction was specific and reproducible (Fig. 2B). The apparent increase in the abundance of a 198 kDa tyrosine-phosphorylated molecule following stimulation, as seen in Fig. 2A, was frequently, but not always, observed (Fig. 2B). Although a tyrosine-phosphorylated protein of approximately the same electrophoretic mobility of SPK89 was observed in these analyses, we have no data concerning of the identity of this phosphoprotein. The absence of any striking regulation in the extent of total protein tyrosine phosphorylation in semi-syn-

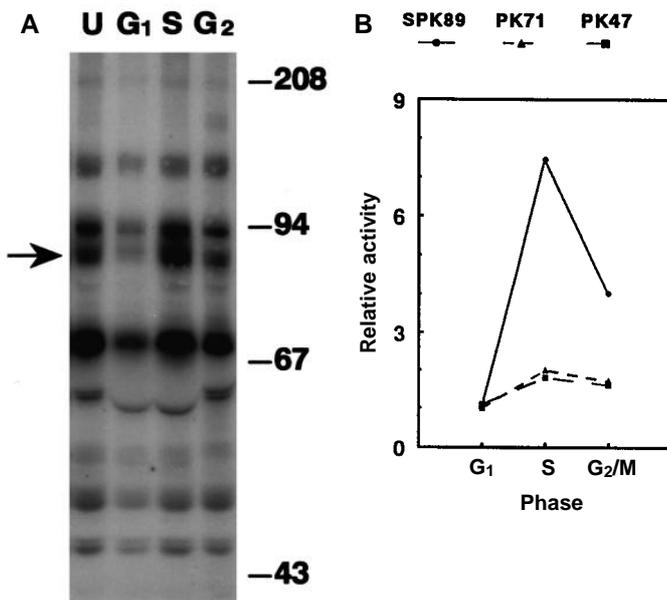


**Fig. 2.** Protein-tyrosine phosphorylation in quiescent and cycling cells. (A) Autoradiogram of anti-phosphotyrosine immunoblot of the same semi-synchronous populations as shown in Fig. 1. Each lane contains  $10^7$  cells. Arrow indicates the position of the 77 kDa induced band seen in the stimulated cells. Mobility of molecular mass standards (kDa) are indicated at right. (B) Protein tyrosine phosphorylation in quiescent and stimulated trypanosomes. Stationary phase cells were harvested at 0 (quiescent) or 5 hours post-stimulation in fresh medium, as indicated by 0 or 5 above each respective lane. Autoradiogram shows results of paired samples from 5 independent experiments. Mobilities of molecular mass standards are indicated in kDa. Arrows indicate the 77 kDa species induced in cycling cells.

chronous populations thus contrasts with the specific and dramatic increase in SPK89 activity as resting cells enter the cell cycle.

### Cell cycle compartmentalization of SPK89 activity in asynchronous exponentially growing cells

The specific induction of SPK89 activity as semi-synchronous procyclic form cells move into S phase of the cell cycle



**Fig. 3.** Cell-cycle stage-specific protein kinase activities in sorted procyclic form cells. Results shown were reproduced in two independent experiments. (A) Protein kinase renaturation autoradiogram of sorted cells from a mid-log culture of procyclic form parasites. U, unsorted; G<sub>1</sub>, S and G<sub>2</sub>/M denote sorted cell cycle phases. Positions of molecular mass standards (kDa) are shown at right. Arrow at left indicates position of SPK89. (B) Phosphorimager analysis of cell cycle-specific protein kinase activities. The kinase renaturation blot from Fig. 4A was subjected to phosphorimager analysis of the various protein kinase activities indicated above (identified by their mobility on SDS-PAGE). The G<sub>1</sub> activities were arbitrarily defined as 1.0.

indicates that SPK89 may play a role in trypanosome proliferation. However, entry into cycle from a previous quiescent state may pass through additional cell cycle checkpoints and may not reflect the regulatory events required for cell cycle progression in actively dividing cells. We therefore sought to assess the relationship of SPK89 activity and the cell cycle in unperturbed exponentially dividing procyclic forms, via analysis of purified cell cycle populations.

The unusual morphology and high motility of trypanosomes rendered the use of cell separation techniques based on density centrifugation inapplicable. We therefore turned to flow cytometric cell sorting as a means of isolating pure populations of G<sub>1</sub>, S and G<sub>2</sub>/M phase cells from asynchronous procyclic form cultures. Since PI staining of cells requires permeabilization of the cell membrane, we first tested whether various methods of cell fixation would permit recovery of SPK89 activity upon kinase renaturation assay. The kinase renaturation profile of cells fixed in 50 or 70% methanol was very similar, if not identical, to that of unfixed control cells (data not shown). As methanol fixation instantly kills the cells, it has the added advantage of 'freezing' the parasites in the particular stage of the cell cycle, thereby preventing further cell cycle progression after purification.

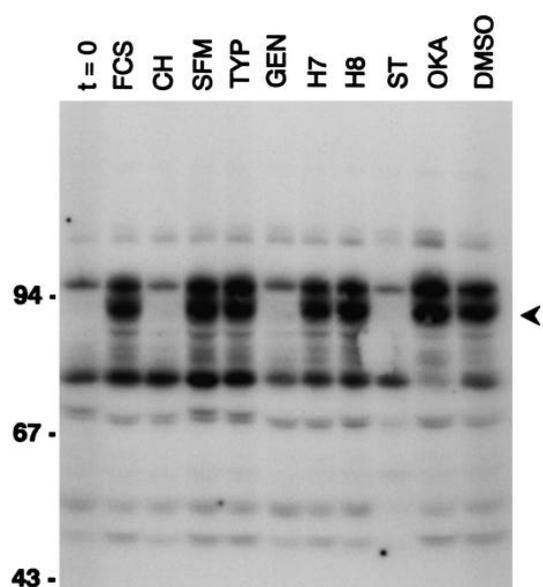
Purified G<sub>1</sub>, S or G<sub>2</sub>/M populations of fixed procyclic form parasites were obtained by cell sorting of PI stained cells. Kinase renaturation assays (Fig. 3A) revealed that the majority

of SPK89 activity was localized to the S phase cell population, with some in the G<sub>2</sub> population. SPK89 activity was low in G<sub>1</sub> phase cells, resembling the level of activity observed in quiescent stationary phase procyclic form parasites and quiescent stumpy bloodforms (Parsons et al., 1993). The relative levels of the several renaturable protein kinases observed in Fig. 3A were quantitated using phosphorimaging and are shown in Fig. 3B, with the G<sub>1</sub> activities arbitrarily defined as 1.0. While the activities of the other renaturable protein kinases were increased by twofold or less in the S phase cells, SPK89 activity increased approximately eightfold, and then dropped by 50% in the G<sub>2</sub>/M population. These data demonstrate that SPK89 activity is highest in S phase of normally cycling cells. PK97 showed a similar but less dramatic pattern of cell cycle regulation in this and similar experiments. However, unlike SPK89, it is not decreased in stumpy bloodforms (Parsons et al., 1993), which are arrested in G<sub>0</sub>/G<sub>1</sub> of the cell cycle (Shapiro et al., 1984).

### Requirements for induction of SPK89 activity and S phase in *T. brucei*

Stationary phase cultures of procyclic form parasites were stimulated by dilution into fresh medium in the presence of various modulators. These inhibitors were used at or above the concentrations known to inhibit enzyme activities in mammalian systems (Miyamoto et al., 1993; Hidaka and Kobayashi, 1992; Gazit et al., 1989). After 5 hours the cultures were harvested and the cells analyzed by the kinase renaturation assay (Fig. 4) and for progression into S phase (Table 1). The induction of SPK89 did not require serum and was not affected by the presence of the protein tyrosine kinase inhibitor tyrphostin A25 or the protein kinase C inhibitors H7 and H8. It should be noted that while H7 inhibits PKC-like activities in *T. brucei* (Keith et al., 1990), the effects of H8 are unknown. The protein phosphatase inhibitor okadaic acid (Haystead et al., 1989) also had no effect on induction of SPK89 activity, though this compound does inhibit *T. brucei* cell division and protein phosphatase 1/2A-like activities in vitro (A. Das and M. Parsons, unpublished observations). Cells treated with H7, H8 or okadaic acid all progressed normally into S phase of the cell cycle. Stimulation with serum-free medium or tyrphostin A25 resulted in a slight delay in entry into S phase, although by 5 hours a shift to S phase was apparent.

Although neither genistein, typically categorized as a tyrosine kinase inhibitor (Akiyama et al., 1987; Linossier et al., 1990), nor staurosporine, a broad spectrum protein kinase inhibitor (Bruno et al., 1992; Hidaka and Kobayashi, 1992), directly inhibited SPK89 activity when included in the kinase renaturation assays (data not shown), both blocked induction of SPK89 in live cells (Fig. 4 and Table 1). In addition, the specific protein synthesis inhibitor cycloheximide prevented the induction of SPK89 activity when added at the time of stimulation. Cells with low SPK89 activity, i.e. unstimulated cells or those treated with cycloheximide, genistein or staurosporine, all failed to progress into S phase (Table 1). Thus, in these experiments inhibition of SPK89 activity correlates with a concomitant block in the trypanosome cell cycle. To determine when cells had synthesized all the components required for cell cycle progression cycloheximide was added to the cultures at various times after dilution into fresh medium. By 3 hours post-stimulation addition of cycloheximide had no



**Fig. 4.** Effect of various modulators on the induction of SPK89 activity. Stationary phase cultures were prepared and stimulated at time 0 by dilution into fresh medium containing the indicated compounds at the concentrations listed below. Autoradiogram shows cells harvested at 5 hours post-stimulation and subjected to the kinase renaturation assay. From left to right, abbreviations for each treatment are as follows: *t=0*, unstimulated stationary phase cells; FCS, medium containing fetal bovine serum (positive control); CH, cycloheximide (71  $\mu$ M); SFM, serum-free medium; TYP, tyrphostin A25 (20  $\mu$ M); GEN, genistein (111  $\mu$ M); H7 (20  $\mu$ M); H8 (50  $\mu$ M); ST, staurosporine (1.0  $\mu$ M); OKA, okadaic acid (500 nM); DMSO, 0.5% dimethylsulfoxide (solvent control). Except for SFM, all media contained 10% fetal bovine serum. Mobilities of molecular mass standards (kDa) are shown at left. Arrowhead denotes position of SPK89.

inhibitory effect on cell growth, with the cells all progressing into S phase at the 5 hour time point (data not shown). Again, SPK89 activity was observed only in cultures with an S phase population. Thus, by 3 hours post-stimulation, all the proteins necessary for progression to S phase, possibly including

SPK89, had been synthesized, thereby rendering the cells growth competent.

#### Staurosporine and genistein inhibit protein synthesis in *T. brucei*

The activities of several protein kinases were also inhibited by growth in staurosporine and genistein (Fig. 4), suggesting that these compounds had wide-ranging effects on cellular processes. Indeed, as shown in Table 1, analysis of  $^{32}$ P-labeled cells revealed that treatment with staurosporine and genistein resulted in a marked reduction in total protein phosphorylation. Both inhibitors also effectively blocked protein synthesis. Genistein- or cycloheximide-treated cells with less than 60% of control protein synthesis did not progress to S phase, even by 10 hours (data not shown). Thus, at concentrations used to study the mammalian cell cycle (Usui et al., 1991), genistein and staurosporine cannot be used as specific protein kinase inhibitors for *in vivo* work with *T. brucei*. Taken together, these data support the concept that protein synthesis is required both for the induction of SPK89 activity and progression from a quiescent state into S phase, but do not rule out a requirement for protein phosphorylation as well.

#### DISCUSSION

In exploring the role of protein kinases in trypanosome development and proliferation, we previously identified a detergent-soluble 89 kDa serine/threonine protein kinase activity restricted to actively proliferating developmental stages of *T. brucei* (Parsons et al., 1993). The present study extends these initial observations by examining the relationship of SPK89 and the trypanosome cell cycle. SPK89 activity was specifically induced as quiescent cells are stimulated to enter the cell cycle, becoming elevated as cells moved into S phase. Similarly, SPK89 activity was localized predominantly to the S phase compartment in exponentially proliferating cells, with some activity residing in the G<sub>2</sub> population. Our studies using various inhibitors further substantiated the claim that induction of SPK89 and S phase are interconnected. We suggest that induction of SPK89 activity may occur very late in G<sub>1</sub> or in early S phase.

**Table 1.** Effects of various treatments on SPK89 activity, cell growth, protein synthesis and protein phosphorylation

Treatment <sup>a</sup>	SPK89 activity <sup>b</sup>	S phase <sup>c</sup>	Proliferation <sup>d</sup>	Protein synthesis <sup>e</sup>	Protein phosphorylation <sup>f</sup>
Control	High	Yes	Normal	Normal	Normal
Cycloheximide	Low	No	No	IC <sub>50</sub> 0.5 $\mu$ M	Slightly reduced
Genistein	Low	No	No	IC <sub>50</sub> 37 $\mu$ M	Inhibited
Staurosporine	Low	No	No	IC <sub>50</sub> 20 nM	Inhibited
Tyrphostin	High	Slight delay	Normal	Normal	Normal
Serum-free	High	Slight delay	One doubling	ND	ND
H7	High	Yes	Normal	Normal	ND
H8	High	Yes	Normal	ND	Normal
Okadaic acid	High	Yes	No	Normal	Normal

<sup>a</sup>Drug concentrations as in Fig. 4.

<sup>b</sup>SPK89 activity from Fig. 4 denoted semi-quantitatively as high or low.

<sup>c</sup>Analyzed by flow cytometry 5 hours post-stimulation.

<sup>d</sup>Cultures were seeded at 10<sup>7</sup>/ml and cell density was analyzed over a 48 hour period of drug treatment. Cell density in the control cultures increased approximately 6-fold.

<sup>e</sup>Measured by TCA-precipitable counts of lysates prepared from cells labeled for 5 hours with [<sup>35</sup>S]methionine.

<sup>f</sup>Determined by SDS-PAGE and autoradiography by lysates from <sup>32</sup>P-labeled cells.

Unlike the 34 kDa CDKs (Draetta, 1990; Lewin, 1990; Nasmyth, 1993) and the 55 kDa Cdc7 (Yoon et al., 1993; Jackson et al., 1993) protein kinases of *Saccharomyces cerevisiae*, which are required for entry into S phase, SPK89 did not require association with a positive regulatory subunit for robust activity in vitro. Of the cell cycle-regulated protein kinases identified thus far, SPK89 appears to be most similar to the *SPK1* gene product of *S. cerevisiae*, which encodes a 90 kDa Ser/Thr/Tyr protein kinase (this molecule is distinct from the MAP kinase-like Spk1 of *Schizosaccharomyces pombe*). The *S. cerevisiae SPK1* transcript is induced as cells progress into S phase (Stern et al., 1993; Zheng et al., 1993). The *spk1* mutation confers a terminal arrest phenotype in which the cells exhibit low viability. These observations indicate that Spk1 is an essential protein kinase, which may function as a positive regulator of DNA synthesis. We propose that SPK89 may be a component of the DNA synthetic regulatory network in trypanosomes, possibly providing a signaling component required for the onset of S phase. It will thus be of interest to determine the relationship between SPK89 activity and that of the candidate trypanosome p34<sup>cdc2</sup> homologues (N. Murphy and J. Mottram, personal communication). Molecular characterization of the SPK89 gene will further our understanding of the regulation of SPK89 activity and its role in the trypanosome cell cycle.

Since cycloheximide prevented the induction of SPK89 activity and S phase in semi-synchronized trypanosomes, it is clear that nutrient-depleted trypanosomes have not synthesized all the essential proteins required for induction of DNA synthesis, perhaps including SPK89 or a positive regulator of SPK89 enzymatic activity. Furthermore, our studies indicate that protein synthesis was only required for a portion of the period preceding S phase in trypanosomes. Thus, similar to START in yeast (Nasmyth, 1993), after a specific interval all necessary proteins were synthesized, thereby allowing the trypanosomes to make the G<sub>1</sub> to S transition.

Despite the fact that African trypanosomes exist in the host multicellular environment, it appears that traditionally defined peptide growth factors are not required for stimulation of stationary phase procyclic forms to progress through S phase. Furthermore, stimulation of resting parasites to enter the cell cycle caused a moderate increase in the extent of tyrosine phosphorylation, but altered the pattern only slightly (most notably by the induction of a 77 kDa species). Together the data indicate that cell cycle-related changes in tyrosine phosphorylation are modest when viewed in the context of total cellular phosphotyrosine-containing proteins. Preliminary analysis of sorted populations supports this hypothesis (unpublished results).

Wheeler-Alm and Shapiro (1992) have previously shown that genistein reduces tyrosine kinase activity in bloodform trypanosome extracts. Surprisingly, we found that both genistein and staurosporine were efficient inhibitors of trypanosome protein synthesis and of total protein phosphorylation (which is primarily serine and threonine phosphorylation in these organisms). Thus these drugs cannot be used as specific protein kinase inhibitors for in vivo studies of trypanosomes. As a result, although genistein and staurosporine inhibit cell cycle progression and SPK89 activity, this cannot be considered as evidence for a direct role of protein phosphorylation in modulating these events. Furthermore, the finding that genistein

inhibits growth of *T. brucei* bloodforms (Wheeler-Alm and Shapiro, 1992) cannot now be attributed to a specific effect on tyrosine kinase activity.

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