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Two proteins involved in kinetoplast compaction

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The kinetoplast is the genome of the single mitochondrion of trypanosomatid Protozoa, and contains up to 30% of total cellular DNA in a network of catenated AT-rich rings. EM studies show that the kinetoplast is organized into a compact, disc-shaped structure in vivo, but little is known about proteins involved in its architecture. Defining such proteins would be useful to understand the molecular biology of this unusual organelle and to design compounds to contain parasite growth. We show here that two proteins, *p1* and *p2* of $M_r \sim 22$ and ~ 21 kDa, respectively, from the trypanosomatid *Crithidia fasciculata* can compact kDNA networks efficiently in vitro, the first such demonstration with purified trypanosome proteins. We show that these proteins are localized exclusively in the parasite's kinetoplast. Our data thus define two proteins potentially involved in kinetoplast organization in vivo.

DNA binding protein; Kinetoplast compaction; Trypanosome; *Crithidia fasciculata*

1. INTRODUCTION

The kinetoplast is the genome of the single mitochondrion of trypanosomatid Protozoa, and may contain as much as 30% of total cellular DNA in a network of catenated AT-rich rings [1]. The rings are of two types, the maxicircles and minicircles, the latter forming the bulk of the network. Electron microscopy (EM) of sectioned parasites suggests that this network is organized into a compact, disc-shaped structure in vivo [2–9]. Isolated networks lose this organization, but may be recompacted with polycations such as spermidine [9]. The role of protein in network compaction is not clear, and it is not known whether cellular polycations, together with special features in the kinetoplast DNA (kDNA), such as the minicircle bend, are in themselves sufficient for viable network organization in vivo. Some evidence nonetheless points to the involvement of protein in kDNA architecture and/or morphogenesis. For example, several small histone-like proteins were identified in the trypanosomatid, *Crithidia fasciculata*, which co-purified with minicircle DNA, protected them from nuclease attack, and were enriched in a mitochondrial fraction [10,15]; and networks obtained from formaldehyde-fixed cells of *C. fasciculata* were de-compacted on exposure to conditions that break protein–DNA cross-links [11].

We show here that two DNA-binding proteins, *p1* (~ 22 kDa) and *p2* (~ 21 kDa) from *C. fasciculata* can compact kDNA networks efficiently in vitro. We show by immunofluorescence microscopy that these proteins are localized exclusively in the parasite's kinetoplast. Our findings thus define two proteins potentially involved in network organization in vivo.

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2. EXPERIMENTAL

2.1. *Parasites*

C. fasciculata, maintained from stocks obtained from Dr. Keith Vickerman (Glasgow), were grown at 27°C in BHI medium (Difco).

2.2. *Protein purification*

Protein was purified from early stationary phase cells essentially as described previously [10]. The procedure included the following successive steps: ammonium sulphate fractionation, gel filtration, and chromatography on heparin–Sepharose, calf thymus DNA–Sepharose and kDNA–Sepharose. In the present work, however, a longer heparin–Sepharose column (10 × 2.5 cm) was used, and the protein was eluted with a 100 ml, 0–2 M gradient of NaCl. This resulted in the separation of *p1* and *p2* from *q1*, *q2*, *r*, and *s* (Fig. 1A).

2.3. *Network compaction*

kDNA networks were extracted and purified essentially as given in Simpson and Simpson [12]. Proteins *p1* and *p2* used for compaction were prepared in two ways. In the first method, purified proteins in kDNA–Sepharose peak fractions (section 2.2; Fig. 1B) were further resolved by SDS-PAGE, and the *p1* and *p2* bands were excised from the gel. Protein in the gel bands was extracted, and renatured in buffer D (0.05 M Tris-HCl, pH 7.9, 20% (v/v) glycerol, 0.1 mg/ml BSA, 0.15 M NaCl, 1 mM DTT, and 0.1 mM EDTA) [13] as described previously [10]. In the second method, kDNA–Sepharose peak fractions containing *p1* and *p2* (Fig. 1B) were pooled and dialyzed against buffer A (25 mM Tris-HCl, pH 7.6, 12% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mM EDTA) before use. To study compaction, networks were incubated at 30°C for 10 min, either separately with gel-extracted/renatured *p1* and *p2* in buffer D, or with a mixture of the dialyzed *p1* and *p2* in buffer A. (There was no detectable difference in size between networks incubated in buffer D or buffer A in the absence of protein). The amounts of DNA and protein used per incubation are given in the legend to Fig. 2. Following incubation, 1 μl ethidium bromide in buffer A was added to give a final concentration of 0.15 μg/ml, and the networks were observed by epifluorescence microscopy within 30

min. This was performed with a Zeiss AXUPHOT microscope equipped with a high resolution video camera coupled to an IBAS image analysis system (Kontron, Germany). Network areas were collected randomly. In the case of uncompacted networks, which retain a disc-shaped appearance and present various angles to the view, only those lying flat were measured.

2.4. *Immunology*

Immunological procedures were as detailed in [10]. Briefly, purified *p1* and *p2* in the kDNA–Sepharose fractions (Fig. 1B) were used to raise polyclonals in rabbits. The polyclonals were processed further as follows. The closely migrating *p1* and *p2* bands from kDNA–Sepharose-purified fractions were separated completely by prolonged electrophoresis, blotted onto nitrocellulose, and were used to adsorb the polyclonals. The adsorbed antibodies were released from the filter by standard procedures [14]. In this way we could select antibodies binding to the *p1* and *p2* protein bands separately. In some experiments the polyclonals were affinity purified against the *p1* and *p2* bands which were not resolved fully. Western blots were developed with alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad).

2.5. *Immunofluorescence microscopy*

A 0.5 ml aliquot of cell culture was spun in a clinical centrifuge for 5 min, the pellet was washed (2 × 1 ml), centrifuged again, and resuspended in 1 ml PBS. 1 μl was spread on 1–2 cm^2 of gelatin coated glass slides and the cells were air dried overnight. They were then immersed in methanol for 1 min, air dried again, and re-hydrated in PBS for 5 min. The cells were incubated for 15 min in normal serum and excess liquid was removed by gentle blotting. Slides were incubated in a humid chamber at 37°C with the selected polyclonal antibodies for 60 min, and washed in PBS (3 × 5 min). Excess liquid was removed and the slides were incubated with anti-rabbit FITC IgG (1:50 dilution in 0.1% BSA in PBS) at 37°C for 30 min. The slides were then washed in PBS and counterstained for 1 min in DAPI (4′,6-diamidino-2-phenylindole; 0.1 μg/ml in 100 mM Tris-HCl (pH 8.0), 100 mM Na_2 EDTA, 1.0 M NaCl). Slides were rinsed in water for 10 min, washed in PBS for 5 min and mounted in 0.1% paraphenylenediamine in 90% (v/v) glycerol and 10% 0.01 M PBS (v/v). Control slides were processed as above except that either a rabbit preimmune serum or different (anti-desmin, anti-α-actinin or anti-laminin) rabbit polyclonal antibodies were used instead of the primary antibodies. The slides were observed using a fluorescene optics microscope (Olympus Vanox-S).

3. RESULTS AND DISCUSSION

Proteins *p1* (~ 22 kDa) and *p2* (~ 21 kDa) were first observed to co-purify with four other DNA-binding proteins, *q1* (~ 17.5 kDa), *q2* (~ 16.5 kDa), *r* (~ 15 kDa) and *s* (~ 13 kDa) [10,15]. *q2*, *r*, and *s*, and also possibly *q1*, are localized largely in the nucleus (Tittawella et al., in preparation). Here we report on *p1* and *p2*, which we have now separated from *q1*, *q2*, *r* and *s* by slightly modifying the original purification protocol (Fig. 1A). Fig. 1B shows *p1* and *p2*, following SDS-PAGE and silver staining, in peak fractions eluting from a kDNA–Sepharose column, the last step in our purification procedure. Fig. 1C shows the strong affinity of these proteins for kDNA, as indicated by retention of minicircle DNA in the gel slot during electrophoresis [10].

p1 and *p2* appear to be closely related proteins. Each protein retained minicircle DNA in the gel-slot in a DNA-binding assay [10]. Although differing in sequence, 15 amino acids from their N-termini are similar in other respects, notably in the unusually high percent-

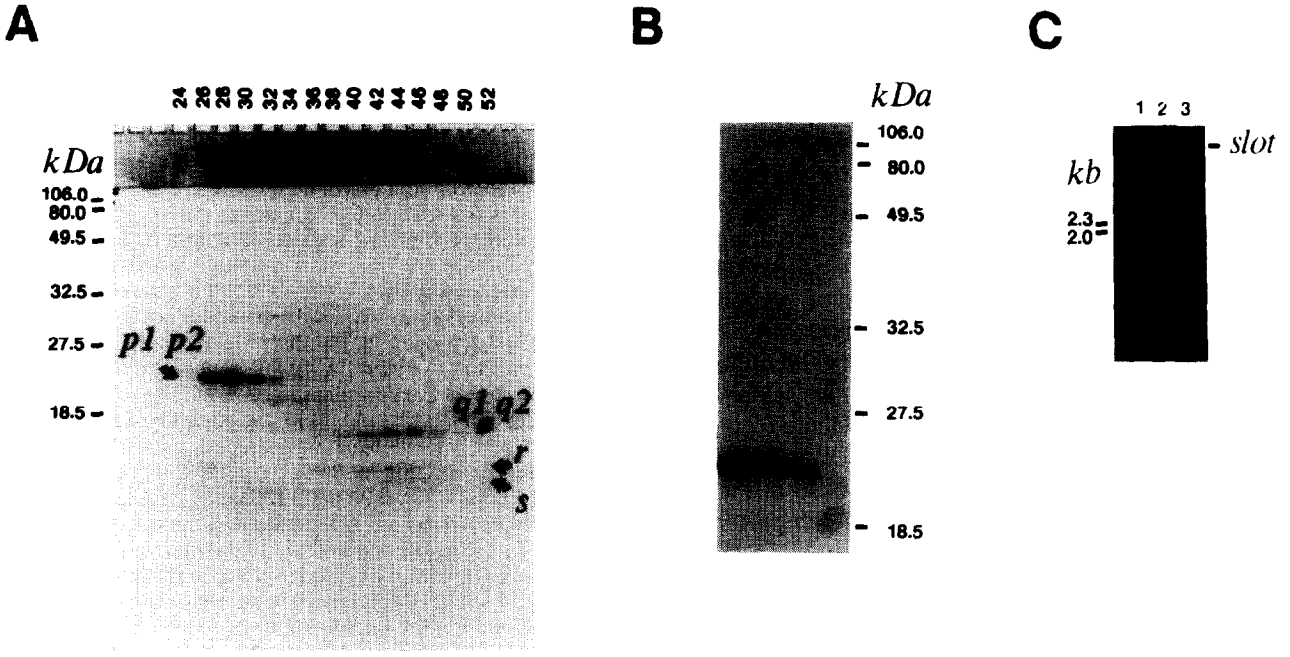

Fig. 1. Purification of *p1* and *p2*. (A) Separation of *p1* and *p2* from *q1*, *q2*, *r* and *s*. For procedures see section 2.2. The figure shows a Coomassie blue-stained 17.5% SDS-polyacrylamide gel in which 50 μl aliquots of fractions eluting from the heparin–Sepharose column were electrophoresed. Fractions are numbered on the top. (B) Material from fractions 28–32 from the heparin–Sepharose chromatogram shown in A were pooled and further purified on calf thymus DNA–Sepharose and kDNA–Sepharose as given in section 2.2. The figure shows three tracks from a silver-stained 17.5–22.5% gradient SDS-polyacrylamide gel containing peak fractions eluting from the kDNA–Sepharose column. (C) Ethidium bromide-stained agarose gel in which ~ 1 μg of *Xba*I-linearized minicircles/track were electrophoresed after incubation in DNA-retention assay conditions [10], with or without purified *p1* and *p2*. Track 1, marker DNA; track 2, minicircle; track 3, minicircle + protein.

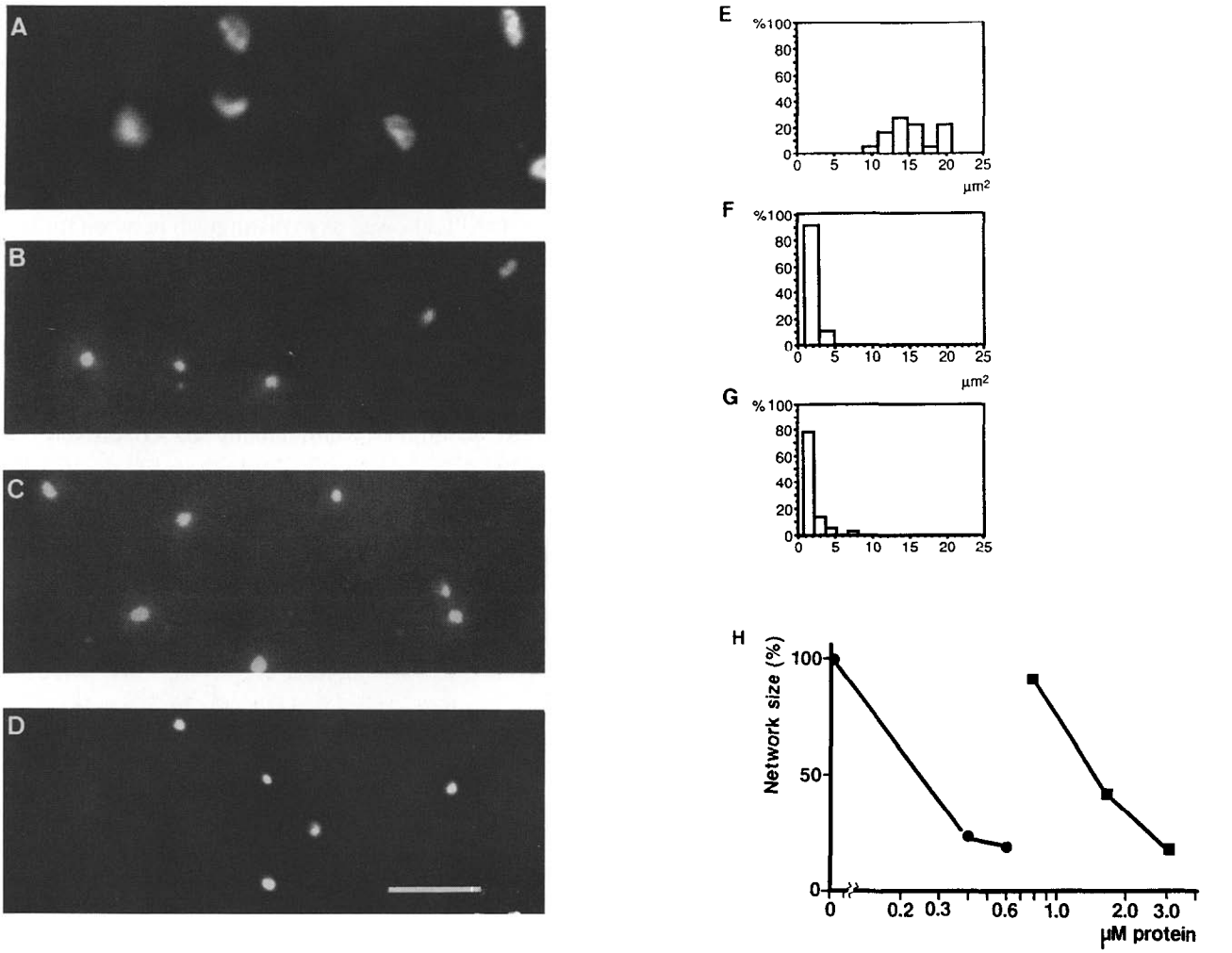

Fig. 2. kDNA network compaction by protein. For details see section 2.3. In all cases 0.8 μg of kDNA networks were used per incubation in a volume of 20 μl. (A–D) Ethidium bromide-stained networks after incubation with or without protein. (For B and C the protein concentration used was not determined). (A) Networks + buffer A. (B) Networks + gel-extracted/renatured *p1* in buffer D. (C). Networks + gel-extracted/renatured *p2* in buffer D. (D) Networks + 0.4 μM of *p1* + *p2* from kDNA–Sepharose peak fractions in buffer A. Bar = 10 μM. (E–G) Size distribution of networks. The percentage of networks measured is plotted against area per network in μm^2. (E) Networks in buffer A; 31 networks were measured. (F) Networks + 0.4 μM *p1* + *p2* in buffer A; 41 networks were measured. (G) Networks + 3.08 μM calf thymus histones (Boehringer) in buffer A; 37 networks were measured. (H) Network size as a function of protein concentration based on data from experiments similar to E–G. In calculating molarity, a M_r of 40 kDa was assumed for *p1* + *p2*, and 130 kDa for the histones. ●, *p1* + *p2*; ■, calf thymus histones.

age (~ 50%) of alanine residues [10]. Thirdly, antibodies selected against carefully separated *p1* and *p2* (section 2.4.) are cross-reactive (see below). The precise nature and biological significance of this relationship are being investigated.

To study the effect of *p1* and *p2* on isolated kinetoplast networks, purified networks were incubated with protein, stained lightly with ethidium bromide, and examined by epifluorescence microscopy. Protein for these experiments was obtained from two sources. For some experiments purified *p1* and *p2* in kDNA–Sepharose peak fractions (fig. 1B) were resolved further by prolonged electrophoresis, recovered from the gel, and renatured after SDS removal (section 2.3.) [10,13]. In other experiments a mixture of *p1* and *p2* in the kDNA–Sepharose peak fractions was used directly. Because of the high degree of variability inherent in SDS removal and renaturation, only a mixture of *p1* and *p2* from kDNA–Sepharose peak fractions was used to quantify compaction efficiency (see below). Fig. 2A shows untreated networks. When treated with either *p1* or *p2* the networks underwent a sharp reduction in size (Figs. 2B,C). A similar compaction was observed when networks were incubated with a mixture of *p1* and *p2* taken directly from kDNA–Sepharose peak fractions (Fig. 2D).

We compared the efficiency of network compaction by *p1* and *p2* in kDNA–Sepharose peak fractions and by calf thymus histones. Fig. 2E shows the size distribution of networks incubated without protein; the bulk of the networks are in the range of 12–20 μm^2. The larger ones probably represent growing networks that were

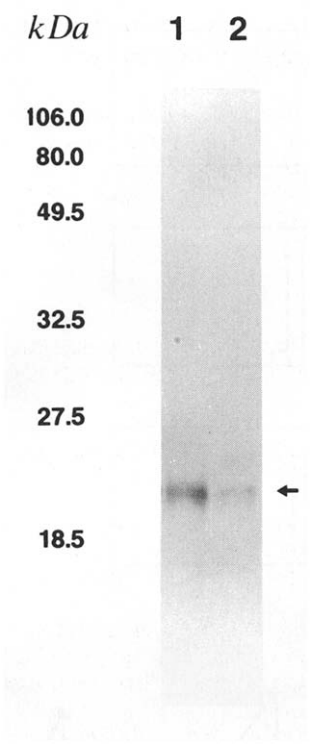

Fig. 3. Western blot of *C. fasciculata* proteins probed with anti-*p1/p2*. See section 2.4. for details. Cells were harvested and processed as given in [10] and the lysate was saturated with ammonium sulphate. The precipitated proteins were dissolved in and dialyzed against buffer A before use. Track 1, ~ 80 μg protein; track 2, ~ 20 μg.

harvested prior to division, while the smallest may include some networks broken during extraction. Fig. 2F and G quantify compaction of the networks after incubation with a mixture of *p1* and *p2* (0.4 μM) and with histones from calf thymus (3.08 μM), respectively. The majority of the networks have undergone > 5-fold reduction in size. In comparison with uncompacted networks this reduction was found to be statistically highly significant, with a P value of < 0.0001, as tested by ANOVA. We cannot yet say if the small minority of networks that fail to compact fully have had a larger starting volume, or were harvested at some stage in their maturation which is relatively resistant to compaction. Fig. 2H shows the mean reduction in network size as a function of protein concentration, summarized from several experiments. Note that the molar concentration of *p1* and *p2* required for maximal compaction of networks was at least six-fold lower than that of calf thymus histones.

We proceeded to localize *p1* and *p2* in cells of *C. fasciculata* by immunofluorescence microscopy. The antibody preparations used in these experiments were either selected against *p1* and *p2* separately, or against the two proteins collectively (section 2.4.). Because of their cross-reactivity, all three antibody preparations gave identical results in Western blots (not shown). A blot of whole *Crithidia* proteins stained with antibody selected collectively against *p1* and *p2* is shown in Fig. 3; as expected, only proteins in the size range of *p1* and *p2* were stained. Control microscopy with either a rabbit pre-immune serum or irrelevant rabbit polyclonals (anti-desmin, anti-α-actinin or anti-laminin) were consistently negative (not shown). Staining with the DNA stain, DAPI, allowed us to distinguish between the more centrally placed nucleus and the kinetoplast, which is located nearest the flagellar base [1,16] (Fig. 4A,B). As shown in Fig. 4C, only the kinetoplast was stained by antibodies selected against *p1* and *p2*. No significant difference in staining pattern was observed in microscopy, whether or not the antibodies used were selected against *p1* and *p2* individually or collectively (not shown).

The strong staining by anti-*p1/p2* suggests that the antigens are bound to minicircles, the main component

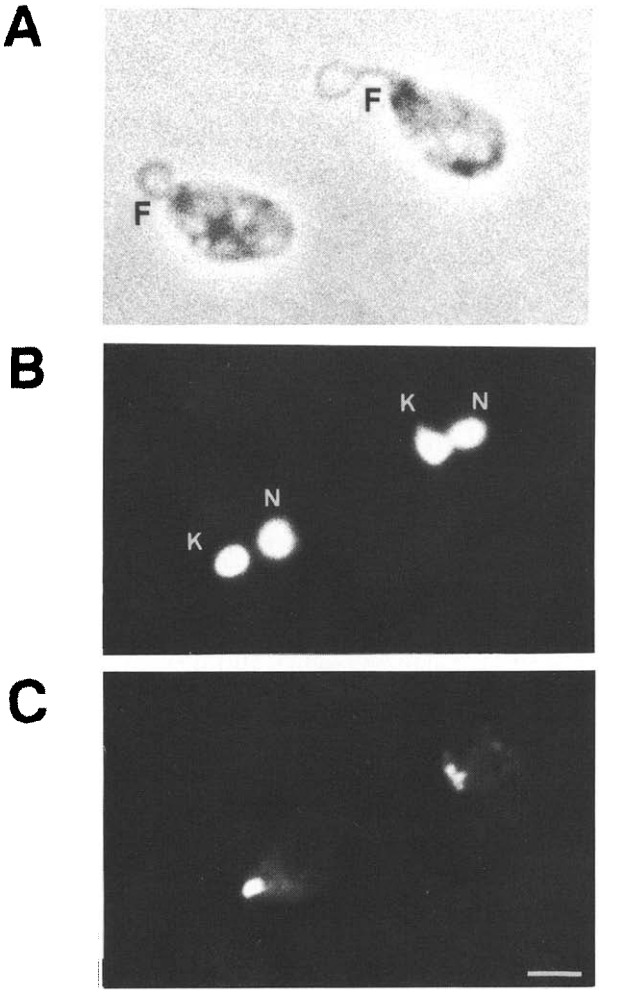

Fig. 4. Immunofluorescence microscopy of *C. fasciculata* cells using affinity-purified anti-*p1/p2* antibodies. The parasites were taken from an asynchronous culture at a cell density of ~ 4×10^7/ml. (A). Two parasites in phase contrast; (B,C) identical pair of parasites stained with DAPI and anti-*p1/p2*, respectively. F, flagellar base; N, nucleus; K, kinetoplast. Bar = 3 μm.

of the kinetoplast [1]; minicircles do indeed co-purify in large amounts with these proteins [10]. Interestingly, in no cell, from either log- or stationary phase, did the area of antibody staining exceed about 50% of the DAPI staining. The antibody staining was also mostly asymmetric with respect to the outline of DAPI staining. This apparent asymmetry varied from one cell to another, due probably to the random manner in which these ovoid parasites settle on the slide during processing. The pattern of staining indicates that the antigens are localized differently than the topoisomerase [17] and the DNA polymerase [8] shown recently to be located at two distinct antipodal sites on the kinetoplast periphery – sites, presumably, of DNA replication [8]. The nature of the DNA in the *p1/p2*-binding regions of the kinetoplast is being investigated.

Our data strongly suggest that *p1* and *p2* are involved in some aspect of kDNA architecture. EM studies show the non-dividing kinetoplast to be organized in a regular, disc-shaped structure in vivo [2–9]. Proteins involved in such organization would be expected to be distributed fairly uniformly in the network. *p1* and *p2*, however, appear to be confined to only part of the kinetoplast (Fig. 4C). This suggests that if *p1* and *p2* compact the network in vivo, the compaction forces operate unevenly within the network. Models proposed for kinetoplast morphogenesis include stages which require uneven compaction, such as when the network constricts prior to division [18]. Proteins involved in such morphogenetic processes may be distributed only in limited areas of the network in vivo. We are now investigating if *p1* and *p2* fall into this category by analyzing more closely their distribution in networks taken from different stages of the cell cycle.

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