Cytoskeletal architecture and components involved in the attachment of *Trypanosoma congolense* epimastigotes

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SUMMARY

Scanning and transmission electron microscopy of *Trypanosoma congolense* epimastigotes attached to a plastic substratum shows them to elaborate a complex flagellum filament system and plaque with a highly organized structure. Non-ionic detergent extraction of these cells shows that the resulting cytoskeletons remain attached to the plaque. The subpellicular corset of microtubules can be removed by salt or Ca^{2+} treatment leaving the axoneme, paraflagellar rod, associated filaments and the plaque. Neither of these treatments therefore removed the plaque-associated material from the substratum. Analysis of these fractions by SDS–polyacrylamide gel electrophoresis reveals an abundant 70 kDa protein that is highly enriched in the salt extracted 'minimal plaque' structures and appears likely to be a major constituent of this structure. These studies reveal that the complex filament and microtubule systems of the cytoskeleton involved the attachment of trypanosomes to substrata and have established a method of biochemical fractionation of the structures and components involved.

Key words: *Trypanosoma congolense*, attachment, cytoskeleton, flagellum, epimastigote, paraflagellar rod.

INTRODUCTION

There are a number of important transformations of cell type that occur during the life-cycle of African trypanosomes (Vickerman, 1985). These transformations and cell type differentiations appear to be intimately linked to cell cycle control since in both the mammalian bloodstream and the tsetse fly stages there occurs a specific alternation of dividing and non-dividing forms of the parasite (Matthews & Gull, 1994). A particularly intriguing cell type is the epimastigote form which multiplies whilst attached to specific areas of the mouthparts of the tsetse fly (Tetley & Vickerman, 1985). This attachment is known to be mediated via an interaction between the flagellum of the parasite and the specific zone of the insect mouth parts. Epimastigote attachment is likely to be a critical phenomenon in the trypanosome life-cycle. Hendry & Vickerman (1988) reported that if *in vitro* attachment was compromised, epimastigote division continued but differentiation to the metacyclic form was prevented. Reports of parasite attachment and development in their invertebrate hosts date back to early light microscopical observations of different species and their location in the host (Ross, 1906; see review by Molyneux, 1977). It is interesting that in all cases studied of trypanosome adhesion in the insect vector, attachment is found to occur only by the flagellum. Early electron microscopical studies by Brooker (1971) of the attachment of *Crithidia fasciculata* to the hindgut of *Anopheles* revealed a large amount of filamentous material in these flagella and their attachment to a dense plaque structure that developed on the flagellar membrane at the region of parasite/host contact. Vickerman (1973) in a study of *T. vivax* attachment to the labrum of the tsetse fly also revealed similar structures and suggested that cells could sometimes adhere to their neighbours via desmosomal-like connections between their respective flagella. A series of studies have emphasized structural changes in the flagellum during attachment in the following systems: *T. brucei* in the salivary glands of the tsetse fly (Tetley & Vickerman, 1985); *Trypanosoma* (*Megatrypanum*) *freitasi* attachment to fibroblasts in culture (Thomaz *et al*. 1992); the attachment of *T. congolense* to bovine aortal endothelial cells (Hemphill & Ross, 1995).

The cellular cytoskeleton of the trypanosome is dominated by a cross-linked subpellicular corset of microtubules which is likely to restrict the construction of local, polarized membrane/cytoskeleton structures are found in systems such as mammalian tissues, where firm cell/surface or cell/cell connections are required (Hynes, 1992; Gumbiner, 1996). Therefore, the use of the flagellar cellular compartment to organize these structures in trypanosomes is interesting. However, in contrast to the mammalian systems we know very little about the identity of molecules, their organization in the flagellar plaque structures, or their construction or removal in attachment/detachment. If we are to understand the importance of these attachments and

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parasite/host interactions in trypanosomatid parasitology, we need to gain an understanding of these processes and molecules. In this study we have focused on the *in vitro* attachment of *T. congolense* epimastigotes since this is likely to be an ideal system in which to allow development of biochemical and molecular approaches. Initially, we have analysed the involvement of cytoskeletal elements in the attachment and describe the biochemical complexity of different elements of the attachment complex providing approaches to the selective enrichment of fractions.

MATERIALS AND METHODS

Trypanosome cultures

Trypanosoma congolense epimastigote forms, stock 1457, were obtained from Dr C. A. Ross, Centre for Tropical Veterinary Medicine, Edinburgh. The cells were grown in Eagles Minimal Essential Medium with Earles salts and 25 mM HEPES (Sigma Chemical Company), supplemented with 2 mM glutamine (Gibco) and 20% foetal calf serum following the method of Gray *et al*. (1981). Cultures were initiated by inoculation of 1 ml of cell suspension containing at least 2×10^6 trypanosomes, into a 25 cm^2 tissue-culture flask. The cells were allowed to settle to the base of the flask, before addition of medium. New cultures were gassed with 5% CO_2 and incubated at 28 °C. The culture medium was changed every 48 h. At these medium changes, the layer of attached cells was washed by expelling the liquid from the pipette vigorously 5–10 times. More firmly adhering trypanosomes were removed by gentle scraping with the pipette end. The cell suspension produced in this way was used to initiate new cultures.

Electron microscopy

For scanning electron microscopy cultures were grown on glass cover-slips in microtitre plates and were washed twice with PBS and fixed in 2·5% glutaraldehyde in PBS for 1 h. The fixative was removed and cells were rinsed with PBS before dehydration through a graded series of acetone/water mixtures, followed by three 1 h passages through 100% acetone. The cover-slips were then critical-point dried, mounted on stubs, coated with gold and examined in a Cambridge 360 SEM.

Cells were processed for transmission electron microscopy by the method of Tooze (1985). Fixation and dehydration was performed with cells still attached to the plastic flask. The final alcohol change was poured off and the cells removed from the base of the flask by briefly adding propylene oxide, which dissolves the plastic. The resultant cells were transferred to Eppendorf tubes, pelleted by a high-speed spin and embedded in Spurr's resin with polymerization at 60 °C overnight. Sections were cut using glass knives and stained in 5% uranyl acetate in 1% acetic acid for 30 min at 60 °C, washed in a stream of distilled water and further stained in lead citrate (Reynolds, 1963) for 10 min at room temperature. After washing, the sections were examined on a Philips 201 microscope.

Polyacrylamide gel analysis

Protein samples were prepared by adding boiling sample buffer (Laemmli, 1970) to the trypanosome pellets and then boiling for 5 min. Gel samples of attached material were made by adding boiling sample buffer into the tissue culture flask and scraping the flask base with a disposable plastic cell scraper. The liquid was then removed and boiled for 5 min prior to loading on gels.

Polyacrylamide (10%) separating gels were run essentially according to the method of Laemmli (1970) on an LKB minigel system. After removal from the apparatus, and fixing in 50% (v/v) methanol, 10% (v/v) acetic acid, gels were stained either by silver staining (Wray, 1981) or Coomassie Blue R250.

Cytoskeletons

Cytoskeletons were produced by extraction of whole cells with either Triton X–100 or Nonidet P-40 (essentially as described by Woods, Baines & Gull, 1989; Robinson & Gull, 1991; Robinson *et al*. 1991). The cytoskeletons were fractionated by the action of calcium ions or by using a high salt concentration (Dolan, Reid & Voorheis, 1986; Schneider *et al*. 1987). These treatments selectively depolymerize the subpellicular microtubules, leaving the flagellum intact.

EGTA was added to the culture at a concentration of 5 mM and the attached trypanosomes were then washed with PMN (10 mM Na_3 PO_4, 150 mM NaCl, 1 mM $MgCl_2$, pH 7·2) buffer. Cytoskeletons were produced by incubation of the cells in 0·5% Triton X–100 in PMN on ice for 5–10 min. The cytoskeletons were washed once in the detergent–buffer mixture, then washed in PMN buffer. Following this, the cytoskeletons were incubated on ice for 45 min in 1 mM $CaCl_2$ in PMN buffer. The salt extraction of cytoskeletons was performed by preparing cytoskeletons *in situ* by incubation in 0·5% Triton in MME buffer (10 mM MOPS (3-[N-morpholino] propanesulfonic acid), pH 6·9; 1 mM EGTA; 1 mM $MgCl_2$) for 5–10 min on ice. The attached cytoskeletons were washed twice in the detergent–buffer solution, before addition of the 0·5 M NaCl in MME buffer. After an incubation period of 5 min, cytoskeletons were detached from the flask base by tapping and were collected by centrifugation.

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RESULTS

Growth of the *T. congolense* culture proceeded by division of the attached epimastigotes on the base of the culture flask. The dividing, attached epimastigotes formed clusters which increased in diameter until large areas of the flask base were carpeted by this population. However, in addition to this population the flasks contained clumps of unattached and single cells. DAPI (4,6-diaminodino-2-phenylimdole) staining and electron microscopy of these cultures showed that the attached cells were mainly epimastigotes (Fig. 1A). The unattached population was more heterogenous showing examples of both epimastigote, procyclic and, in late cultures, metacyclic cellular configurations as judged by DAPI staining.

Scanning electron microscopy of attached trypanosomes grown on glass and plastic cover-slips again showed that they possessed the blunt posterior cell end with attachment to the substratum by the more distal portion of the flagellum, the membrane of which is flattened out into a series of blebs and protrusions (Fig. 1B). *T. congolense* cells attached to the base of a tissue culture flask were then studied by electron microscopy. Our fixation and embedding schedule allows us to maintain cells attached to the substratum until late in the embedding procedure and then view them in transverse section. Hence, we were able to define the attachment zones on the thin electron-dense lines of material that presumably represent the culture extracellular matrix proteins that had formed a thin cover on the plastic substratum. Examination of thin sections revealed that profiles of attached cells were arranged along these lines of electron-dense material, derived from the base of the flask (Fig. 1C and D). All trypanosomes were attached via their flagella and commonly, they were seen in rows (Fig. 1D) with their flagella positioned along the flask base. The membrane of the flagellum is flattened along the line of attachment and condensed on the inner side of the flagellar membrane, in contact with the substratum, is an electron-dense layer of material resembling a hemidesmosome plaque (Fig. 1D). Short connecting filaments from the plaque link up with the nearest group of the outer microtubule doublets of the flagellar axoneme and filaments encircle the axoneme and the paraflagellar rod (PFR), extending to the flagellum attachment zone (FAZ).

To investigate the nature of the attachment, trypanosomes were subjected to a detergent treatment to remove the membranous components of the cell. If adhesion were solely mediated by specific membrane components in contact with the substratum, attachment might be disrupted by stripping away the membranes. *In situ* detergent extraction of trypanosomes, however, did not disrupt their attachment. TEM examination of cytoskeletons produced in this way (Fig. 2A) revealed that most of the membranes had been removed and the layer of plaque material lined the areas of cytoskeleton attachment. Although some of the fibrous network of filaments encircling the axoneme and the paraflagellar rod had been removed, the majority of attached cytoskeletons retained some degree of plaque associated filamentous material. The doublets most often found in the vicinity of the plaque were identified as doublets 1 and 2 and their outer neighbours 9 and 3 respectively. This positioning of these doublets nearest the plaque is likely if one considers the precise construction of the trypanosome cytoskeleton (see Bastin, Matthews & Gull, 1996). The PFR is linked to the axoneme at the B-subfibre of doublet 7. The PFR then connects with the internal face of the flagellar membrane in the region of the FAZ. Doublets 9–3 lie opposite the PFR and doublet 7 and therefore are closest to the plaque.

The stability of attachment in the face of detergent extraction emphasized the importance of the links between the internal cytoskeleton and the plaque in maintaining adhesion. We then asked if the attachment system was organized and linked to the whole of the trypanosome cytoskeleton (microtubule corset and flagellum), or, if the microtubule corset could be stripped away without affecting attachment, leaving behind an attached flagellum with associated plaque. This was tested using the technique of calcium treatment to selectively depolymerize the pellicular microtubules leaving isolated flagella (Fig. 2B). Treatment of attached cytoskeletons with calcium destroys the microtubule corset, leaving isolated flagella adhering to the substratum (Fig. 2B). The attached flagella retain the FAZ, clearly visible in longitudinal section (Fig. 2C). As before, the plaque material persists beneath the attached flagella and accessory filaments are present in varying quantity. Short connections extend from the plaque to the axoneme of attached flagella. In Fig. 2B, the axoneme is moored in position by links between the plaque and the microtubule doublets 1, 2 and 3, which are closest to the plaque. At the opposite side of the axoneme, the customary linkage from doublet 7 to the PFR was present and the PFR was connected to the flagellar membrane juxtaposed to the cell body. Hence, the microtubule corset of the cell body can be removed successfully without affecting the integrity of attachment, leaving isolated flagella anchored above the plaque by connections to the axoneme.

We then studied the effect of the high salt concentrations which are also known to depolymerize the subpellicular microtubules. NaCl at 0·5 M was found to be the lowest salt concentration effecting removal of cell body cytoskeletons and solubilization of the subpellicular microtubule corset. Transmission electron microscopy of the at-

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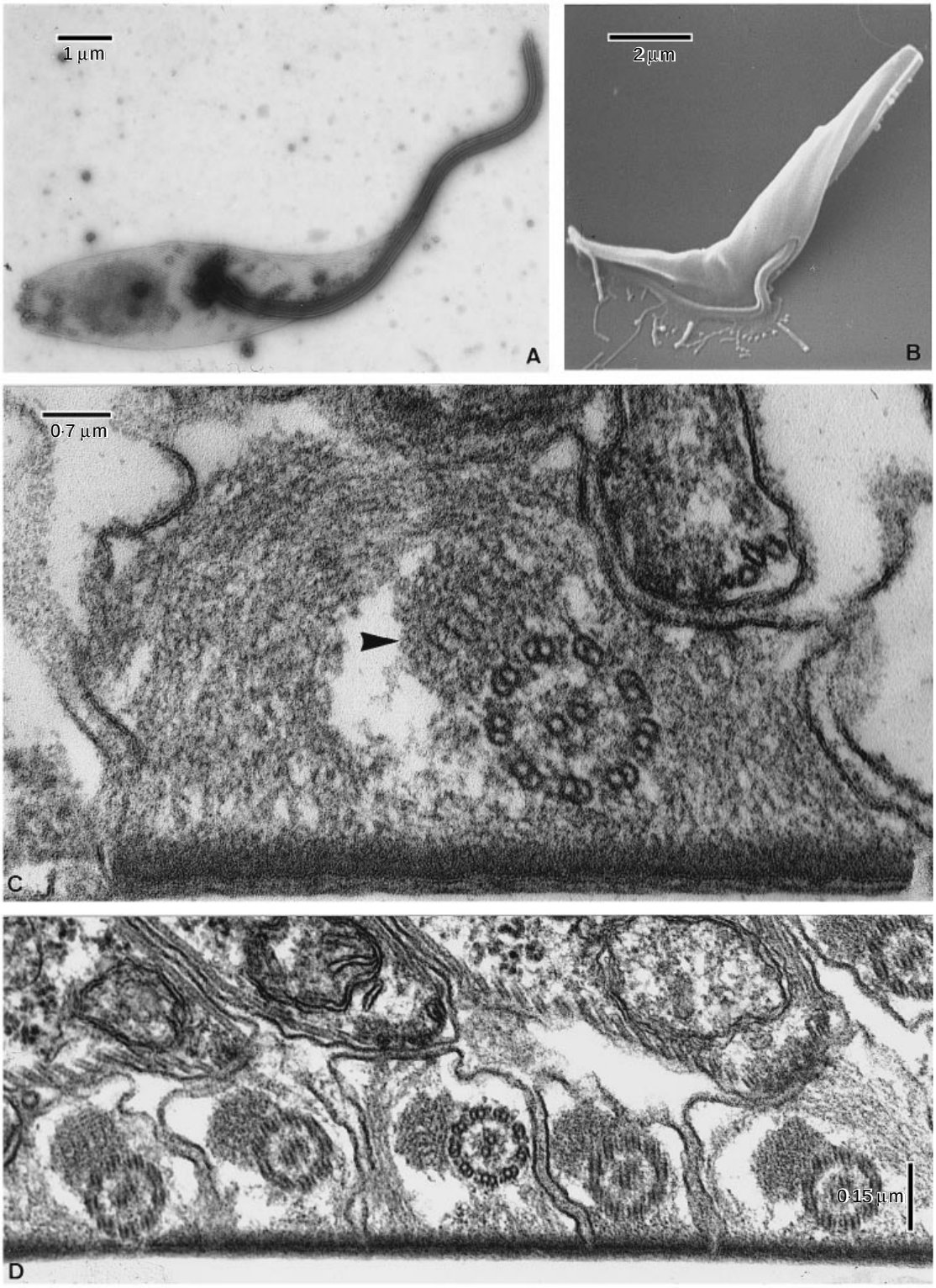

Fig. 1. (A) Negatively stained detergent extracted cytoskeleton of *Trypanosoma congolense* epimastigote showing features of posterior nucleus and anterior basal body. (B) Scanning electron micrograph of an attached epimastigote. The region of contact with the substratum is the flagellum membrane which has become expended into blebs and protrusions. (C) Transverse section through the flagellum of an attached cell. The flagellum membrane is attached to the substratum with an electron-dense plaque structure. The matrix of the flagellum is filled with a profusion of filaments that surround the axoneme and the paraflagellar rod (arrowhead). (D) Transverse section through the flagella of five attached cells. Note the similar axoneme and PFR configuration. Plaque material lines the flagellar membrane in contact with the substratum and filaments are present in the flagellar matrix.

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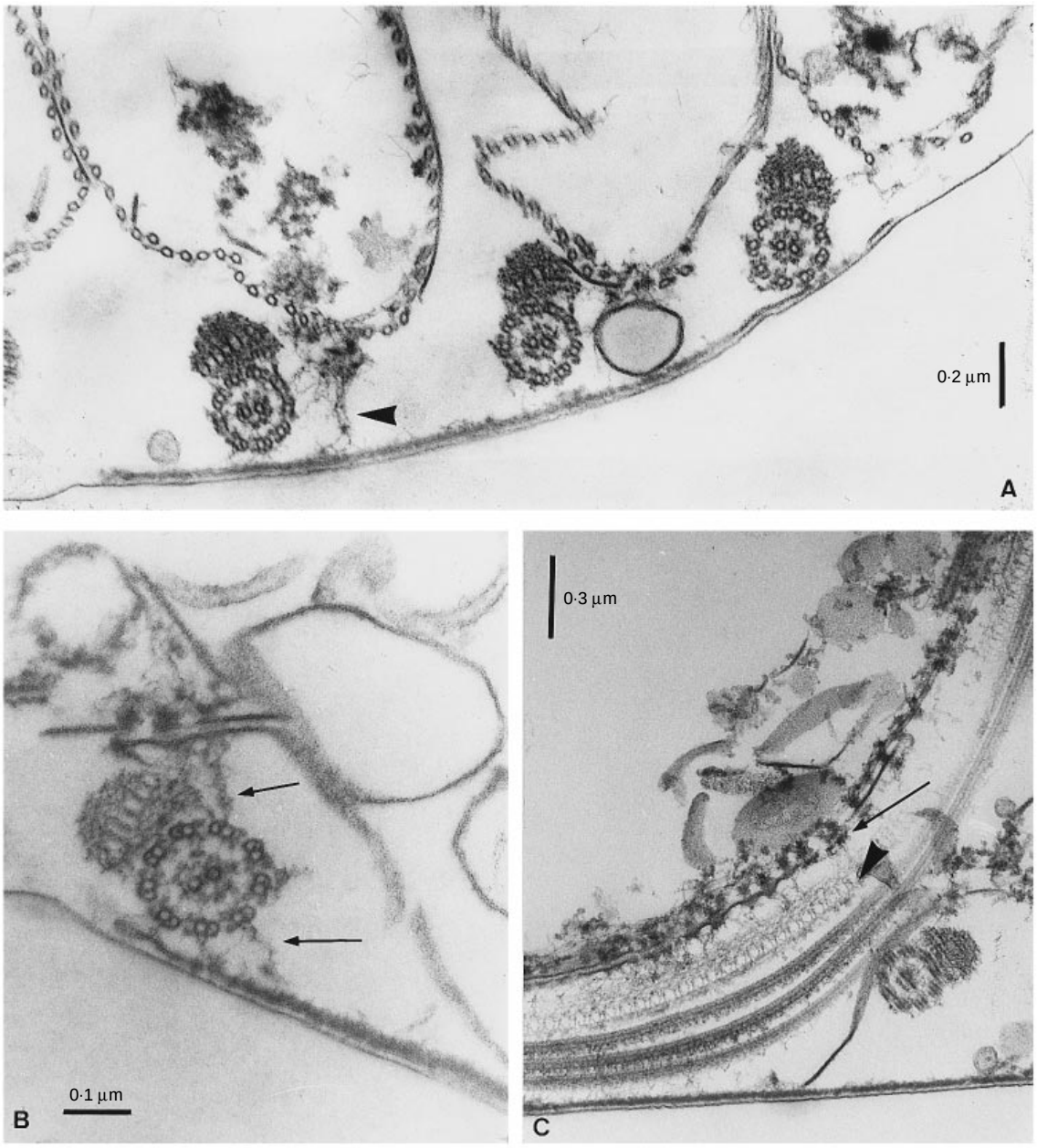

Fig. 2. (A) Transverse section of attached cytoskeletons. Note the electron-dense links (arrowhead) between the electron-dense plaque and the flagellum axoneme. (B) Thin section through an attached cytoskeleton. The substratum area beneath the flagellum is lined with a deposit of plaque material. Note the filamentous connections between the plaque and the axoneme doublets and the axoneme doublets and the remnants of membrane (arrows). (C) Longitudinal section through an attached flagellum. The lower portion of this section has fortuitously included the punctate electron-dense flagellum attachment zone filament complex (small arrow), the links through to the paraflagellar rod (arrowhead) and then a portion of the axoneme.

tached material remaining on the substratum revealed areas of electron-dense plaque material which were sometimes still attached to flagella axonemes and PFR structures. 'Footprints' of localized plaque material and fine electron-dense filaments were located between the flagella and the substratum. There is no evidence of any membranous material remaining at this stage (Fig. 3A and B). This high salt treatment solubilized the outer doublets in a few axonemes. However, even in these cases the plaque and associated filaments remained (Fig. 3A). These images have been chosen to illustrate the range of residual structures seen i.e. PFR and axoneme plus plaque; axoneme plus plaque; axoneme remnant plus plaque and plaque only. Surveys of sections of this material, however, indicate a vast preponderance of 'plaque only' profiles. This is an important point when analysing the polyacrylamide gel profiles (see below).

The Ca^{2+} and NaCl treatments of attached cytoskeletons were similar in that both remove the cell body microtubule corset but neither detached the

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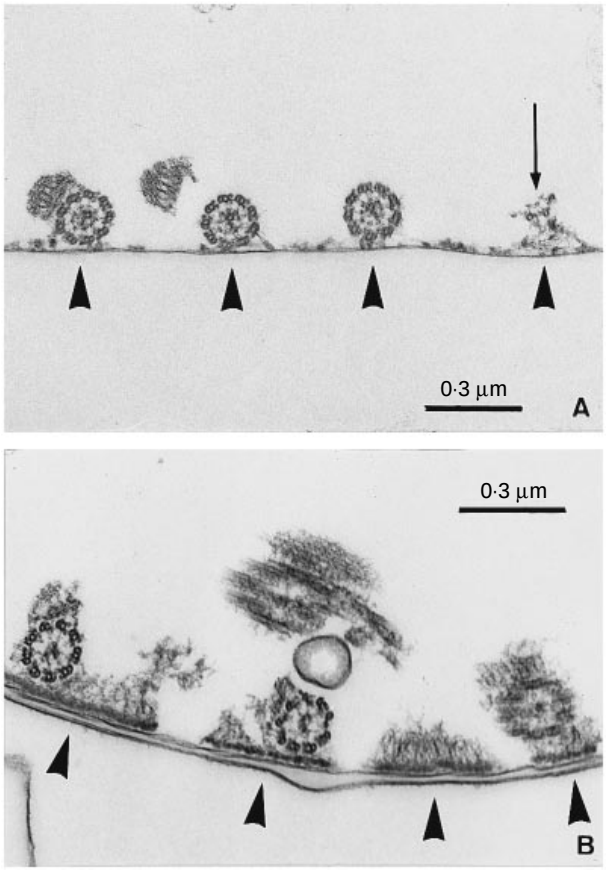

Fig. 3. (A) Transmission electron micrographs of the flask base following NaCl treatment to remove the bulk cytoskeletal material. The large arrowheads indicate the remaining plaque material with associated axonemes. Note (small arrow) that in one area, even though the treatment has removed the outer doublets from the axoneme, the central pair microtubules are still associated with filaments and plaque material. (B) Cross section of NaCl treated material to show the plaque material (arrows) deposited in association with the substratum and the flagellar axoneme. Note that the *in situ* fixation protocol produces a thin electron-dense line of material from the flask surface. In this case the layer has become folded back and therefore appears to be a double line. Both (A) and (B) are used to illustrate the range of structures seen to be attached to the flask. However, there was a preponderance of plaque-only material when sections were scanned.

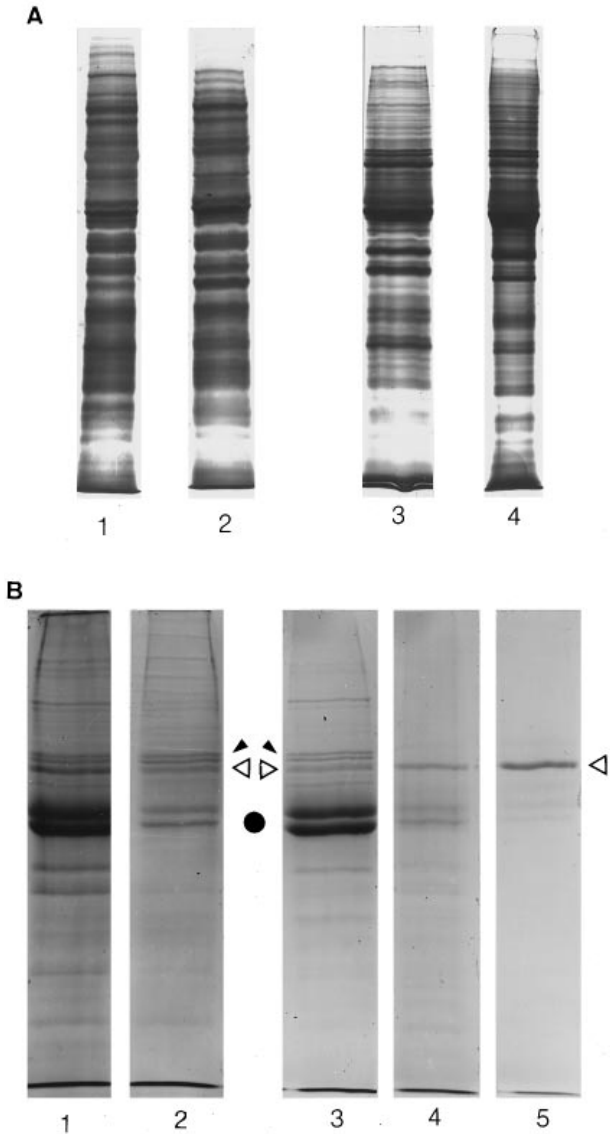

Fig. 4. (A) Silver-stained SDS–polyacrylamide gel proteins of trypanosome populations attached to the growth flask (lane 1) and unattached (lane 2) in the culture supernatant. Lanes 3 and 4 show silver-stained gels comparing detergent-insoluble cytoskeletons of trypanosomes attached (3) and unattached (4) to the flask during growth of the *Trypanosoma congolense* culture. (B) Coomassie blue staining of proteins separated by SDS–PAGE of (1) attached cytoskeletons; (2) attached flagella after Ca^{2+} treatment; (3) cytoskeletal material released by salt treatment; (4) proteins solubilized during salt treatment; (5) proteins remaining on flask surface after salt treatment. (●) Indicates the tubulin doublet, (▶) the PFR doublet, and (◁) the 70 kDa protein.

plaque from the substratum. However, the NaCl treatment appeared more stringent in that a very clean footprint of the plaque material was left together with a set of fine filaments linking the axoneme and PFR to the plaque. There was no evidence of any flagellar or cell body membrane remaining after this detergent/high salt treatment. This treatment of cytoskeletons therefore appeared to leave the minimum footprint of plaque/filament/axoneme/PFR attachment complex and in many cases seemed to leave only the plaque material (Fig. 3B).

We performed an SDS–PAGE analysis of the proteins associated with these fractions. An initial comparison of cells and detergent-extracted cytoskeletons of the attached and unattached cell populations showed very similar patterns (Fig. 4A). However, we then analysed the proteins present in the fractions that remained attached after detergent/Ca^{2+} or detergent/salt treatment. Fig. 4B shows the main proteins of the attached cytoskeletons and the most abundant proteins are the tubulins together

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with a triplet of bands of higher molecular weight and a doublet of bands of rather lower molecular weight. The identity of these bands was confirmed by Western blotting with specific monoclonals (data not shown). The triplet of bands is composed of the 2 doublets of the paraflagellar rod proteins (75 and 72 kDa) and a lower band of around 70 kDa. After Ca^{2+} treatment of these attached cytoskeletons, the material remaining on the substratum had the SDS-gel patterns of major protein bands typical of isolated flagella (tubulin doublet and PFR doublet) along with the 70 kDa protein band (Fig. 4B). An analysis of cytoskeletons detached from the substratum by salt treatment and recovered by centrifugation showed prominent tubulin bands together with more minor amounts of the PFR doublet bands and the 70 kDa band. The proteins solubilized by NaCl (Fig. 4B) revealed small amounts of tubulin but rather more of the 70 kDa protein. However, analysis of the material left on the flask surface after salt treatment showed a massive enrichment of the 70 kDa band (Fig. 4B, lane 5) with only very minor amounts of the PFR and tubulin protein bands. A comparison of relative intensities of protein bands in Fig. 4B lane 5 with the electron micrographs shown in Fig. 3 clearly indicates that the 70 kDa protein must be a major component of the plaque structure since this is the major residual protein of the attached complex, reflecting the abundance of the residual plaque structures (see above).

DISCUSSION

There is extensive evidence that attachment of trypanosomes to cell surfaces and other sites is mediated via the flagellum. Since attachment signifies a change from the motile to non-motile state, it seems rather strange that the very organelle providing the active movement is the one used for this attachment. The attachment cycle of trypanosomes which appears to be so critical in their digenetic life-cycle presumably involves 4 major phases; an initial surface recognition process, a subsequent tethering process and then a consolidation process that leads to the development of the full attachment complex and finally a release process.

An explanation for the use of the flagellum for attachment may lie in the need to provide surface mobility and clustering of receptors which may be more easily achieved on the flagellum membrane than on the main cell body which has a highly structured subpellicular array of microtubules. Such clustering is known to be important for development of adhesive strengthening after initial attachment in mammalian cell systems (Gumbiner, 1996) and it is known that mobility of surface recognition molecules is possible on flagella of organisms such as *Chlamydomonas* (Homan *et al*. 1987). Moreover, the formation of the extensive filament/plaque system (tethering and consolidation?) may be impeded by the subpellicular microtubule architecture in the cell body but may be permitted in the flagellar membrane context. Subsequent work on the early attachment events may provide information relevant to this debate.

Our work has focused on the later events in attachment involving the nature and components of the plaque. The ultrastructural observations made here of a cytoplasmic plaque, a series of filamentous links and flagellar membrane extensions are in agreement with reports in the literature on the attachment of trypanosomes *in vivo* and *in vitro* (Evans, Ellis & Stamford, 1979; Gray *et al*. 1981; Prain & Ross, 1990). In addition, we note in this study that the majority of cells conform to a particular orientation whilst attached. The constant position of the PFR alongside the flagellar axoneme, linked to microtubule doublets 4 and 7 and its connection to the cell body, place doublets 9, 1, 2 and 3 closest to the adhesion plaque. This type of configuration is also seen in the attached trypanosome illustrated in the review by Vickerman *et al*. (1988). It is possible that this is the most favourable position adopted by the attached trypanosome and the connections between certain doublets of the axoneme and the plaque may be important in forming the foundations for construction of the attachment complex.

Extraction of attached epimastigotes with non-ionic detergent does not disrupt their attachment and they remain adhering to the substratum. A previous report by Vickerman *et al*. (1988) that showed protein gel profiles of attached cytoskeletons, provided some evidence of the resistance of the trypanosome attachment complex to the effects of non-ionic detergents used to produce cytoskeletons. The data presented here correlate a biochemical description with the ultrastructure of the attached cytoskeletons. The material composing the plaque is clearly of a nature that resists disruption and solubilization by non-ionic detergent. The accessory filaments associated with the plaque also survive this treatment and remain in place to link the microtubular cytoskeleton to the attachment plaque.

Subjecting attached epimastigotes to extraction, followed by calcium treatment (Dolan *et al*. 1986) produces isolated attached flagella, which retain the adhesion plaque and a complement of filaments. It appears from these studies that the plaque is not directly linked to the cell body microtubule corset, but is linked directly to elements of the flagellum, particularly the axoneme. Of interest in these micrographs is the spatial overlap of the filaments from the FAZ with those emanating from the attachment plaque, suggesting the possibility of direct linkage between the FAZ filaments and the attachment plaque. This idea has been alluded to previously, in a report of attached whole cells of *T*.

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congolense in vitro (Vickerman *et al*. 1988). This proposal is enlarged upon in this study, where an association is seen between the two sets of filaments in TEM investigations through the range of attached whole cells, cytoskeletons and flagella. It is not possible at this stage to rule out the possibility that this represents merely a fortuitous spatial overlap of filaments within the attached flagellum. Nevertheless, it is intriguing to speculate that the filaments of the two systems are associated in some way. So far, the identity of the filaments composing each of these structures is unknown. Such a connection would presumably strengthen the cell body–flagellum adhesion in the attached cell under duress of fluid flow in the mouthparts of the tsetse fly.

In the experiments outlined here, we find an abundant 70 kDa protein in fractions of attached cytoskeletons and flagella. This protein is the most abundant protein remaining on the substratum following high salt treatment, which removes most of the cellular material leaving behind isolated plaques and remnants of axonemes in some cases. The results obtained here differ from a report by Vickerman *et al*. (1988) who found using 2-D gel electrophoresis that attached cytoskeletons of *T. congolense* possessed a novel 70 kDa protein, which was absent from unattached counterparts. Initially, it was puzzling that in our studies this protein was detected in both attached and unattached cells and cytoskeletons. However, the explanation probably lies in differences between the culture systems used. Unfortunately, there are no details of the cell types present in the unattached population from the work discussed by Vickerman *et al*. (1988). We have analysed the unattached population from our cultures by DAPI staining and demonstrated the presence of bundles of epimastigotes which adhere to one another forming rosettes. It is likely that these may be attached by plaques equivalent to those seen in the cells attached to the substratum. It has been shown that the rosettes of cells present in the medium of *Crithidia fasciculata* cultures are maintained by attachment plaques formed between the flagella of neighbouring cells (Brooker, 1970). It was also observed in these cultures that cells were commonly found adhering to particles of debris remnants of dead cells present in the culture system. Thus in our system it is likely that the 'unattached' cell population from the *T. congolense* culture system in fact contains a large amount of plaque material. Given this, it appears that our detection of the 70 kDa protein in the salt-cleaned plaques suggests that this protein is the major constituent of the attachment plaque. Our salt extraction of the cytoskeletons that leaves the plaque/axoneme attached to the substratum provides an opportunity for future immunological/molecular approaches to the molecular characterization of attachment plaque components in trypanosomes.

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REFERENCES

BASTIN, P., MATTHEWS, K. R. & GULL, K. (1996). The paraflagellar rod of kinetoplastida: solved and unsolved questions. *Parasitology Today* **12**, 302–307.

BROOKER, B. E. (1970). Desmosomes and hemidesmosomes in the flagellate *Crithidia fasciculata*. *Zeitschrift für Zellforschung* **105**, 155–165.

BROOKER, B. E. (1971). Flagellar attachment and detachment of *Crithidia fasciculata* to the gut wall of *Anopheles gambiae*. *Protoplasma* **73**, 191–202.

DOLAN, M. T., REID, C. G. & VOORHEIS, H. P. (1986). Calcium ions initiate the selective depolymerisation of the pellicular microtubules in bloodstream forms of *Trypanosoma brucei*. *Journal of Cell Science* **80**, 123–140.

EVANS, D. A., ELLIS, D. S. & STAMFORD, S. (1979). Ultrastructural studies of certain aspects of the development of *Trypanosoma congolense* in *Glossina morsitans morsitans*. *Journal of Protozoology* **26**, 557–563.

GRAY, M. A., CUNNINGHAM, I., GARDINER, P. R., TAYLOR, A. M. & LUCKINS, A. G. (1981). Cultivation of infective forms of *Trypanosoma congolense* from trypanosomes in the proboscis of *Glossina morsitans*. *Parasitology* **82**, 81–95.

GUMBINER, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345–357.

HEMPHILL, A. & ROSS, C. A. (1995). Flagellum-mediated adhesion of *Trypanosoma congolense* to bovine aorta endothelial cells. *Parasitology Research* **81**, 412–420.

HENDRY, K. A. K. & VICKERMAN, K. (1988). The requirement for epimastigote attachment during division and metacyclogenesis in *Trypanosoma congolense*. *Parasitology Research* **74**, 403–408.

HOMAN, W., SIGNON, C., VAN DEN BRIEL, W., WAGTER, R., DE NOBEL, H., MESLAND, D., MUSGRAVE, A. & VAN DEN ENDE, H. (1987). Transport of membrane receptors and the mechanics of sexual cell fusion in *Chlamydomonas eugametos*. *FEBS Letters* **215**, 323–326.

HYNES, R. O. (1992). Integrins: versatility, modulation and signalling in cell adhesion. *Cell* **69**, 11–25.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly stage of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MATTHEWS, K. R. & GULL, K. (1994). Cycles within cycles: the interplay between differentiation and cell division in *Trypanosoma brucei*. *Parasitology Today* **10**, 473–476.

MOLYNEUX, D. H. (1977). Vector relationships in the Trypanosomatidae. *Advances in Parasitology* **15**, 1–82.

PRAIN, C. J. & ROSS, C. A. (1990). *Trypanosoma congolense*: the appearance and distribution of variable antigen types during metacyclic differentiation *in vitro*. *Parasitology* **100**, 107–113.

https://doi.org/10.1017/S0031182097001042 Published online by Cambridge University Press

REYNOLDS, E. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

ROBINSON, D. R., BEATTIE, P., SHERWIN, T. & GULL, K. (1991). Microtubules, tubulin and microtubule-associated proteins of trypanosomes. *Methods in Enzymology* **196**, 285–299.

ROBINSON, D. R. & GULL, K. (1991). Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. *Nature, London* **35**, 731–733.

ROSS, R. (1906). Notes on the parasites of mosquitoes found in India between 1895 and 1899. *Journal of Hygiene* **6**, 101–109.

SCHNEIDER, A., SHERWIN, T., SASSE, R., RUSSELL, D. G., GULL, K. & SEEBECK, T. (1987). Subpellicular and flagellar microtubules of *Trypanosoma brucei* contain the same alpha-tubulin isoforms. *Journal of Cell Biology* **104**, 431–438.

TETLEY, L. & VICKERMAN, K. (1985). Differentiation in *Trypanosoma brucei*: host–parasite cell junctions and their persistence during acquisition of the variable antigen coat. *Journal of Cell Science* **74**, 1–19.

THOMAZ, N., SOARES, M. J., DE SOUZA, W. & DEANE, M. P. (1992). Ultrastructural aspects of *Trypanosoma* (*Megatrypanum*) *freitasi* in co-cultivation with mammalian cells. *Parasitology Research* **78**, 209–214.

TOOZE, J. (1985). Blocked coated pits in At 20 cells result from endocytosis of budding virions. *Journal of Cell Biology* **101**, 1713–1720.

VICKERMAN, K. (1973). The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: an ultrastructural study of the epimastigote stage of the trypanosome. *Journal of Protozoology* **20**, 394–404.

VICKERMAN, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *British Medical Bulletin* **41**, 105–114.

VICKERMAN, K., TETLEY, L., HENDRY, K. A. K. & TURNER, C. M. R. (1988). Biology of African trypanosomes in the tsetse fly. *Biology of the Cell* **64**, 109–119.

WOODS, A., BAINES, A. J. & GULL, K. (1989). Evidence for a M 88000 glycoprotein with a transmembrane association to a unique flagellum attachment region in *Trypanosoma brucei*. *Journal of Cell Science* **93**, 501–508.

WRAY, W., BOULIKAS, T., WRAY, P. & HANCOCK, R. (1981). Silver staining of proteins in polyacrylamide gels. *Analytical Biochemistry* **118**, 197–203.

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