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MICROTUBULE ASSOCIATED PROTEINS (MAPS) – A POSSIBLE CHEMOTHERAPEUTIC (OR IMMUNOLOGICAL) TARGET AGAINST TRYPANOSOMES

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SPECIFIC AIMS AND RESULTS

Aims

A. To isolate microtubule associated proteins (MAPs) which are unique to the trypanosome.

B. To screen various trypanosome species for their MAP content.

C. To check whether these MAPs can serve as targets for chemotherapeutic or immunological suppression of trypanosomiasis.

Results

A. Two unique subpellicular MAPs were isolated from *Trypanosoma brucei* which have a molecular weight of 52kDa (p52) and 15kDa (p15). These proteins were identified as MAPs because of their ability to bind tubulin *in vitro*, to promote tubulin polymerization and to crosslink microtubules to a structure similar to the original form of the trypanosome subpellicular microtubules. Antibodies directed against these proteins labeled the subpellicular microtubules specifically and had no crossreaction with mammalian microtubules, thus indicating their uniqueness to trypanosomes.

B. These proteins were found also in *T. rhodesiense* and in *T. evansi*, suggesting that these proteins may be a common therapeutic target for all trypanosome species.

C. Preliminary vaccination experiments showed that when rodents were vaccinated with p52 along with aldolase and GAPDH (which copurified with p52), 100% protection was achieved against a challenge of a *T. brucei* infection. Several trials to repeat these results were unsuccessful. However, sera of vaccinated and protected rats caused the agglutination of parasites *in vitro*, and antibodies were found within the parasites, thus suggesting that intracellular antigens may indeed serve as immunological target sites for the suppression of trypanosomiasis.

Collaboration with the Kenya Trypanosomiasis Research Institute (KETRI)

The research program was discussed with Prof. A.R. Njogu, Director of KETRI,who designated Mr. Henry Waithaka as the collaborating research and technical assistant for the project.

Henry Waithaka spent two periods of two and a half months each at the Weizmann Institute of Science working closely with Naomi Balaban – August to October 1987 and 1988.

He participated in the research and was introduced to the biochemical techniques used in our laboratory. During his first visit he introduced techniques for growing *Trypanosoma brucei* and participated in the development of the methods for isolating the subpellicular microtubules.

During his second visit he participated in the continuation of the vaccination experiments initiated by Naomi Balaban at KETRI.

Naomi Balaban worked at KETRI for two and a half months during April to June 1988. During that visit antigens for vaccination were purified and vaccination experiments were done in collaboration with the Biochemistry Section at KETRI.

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SUMMARY

The cell body of Trypanosomatidae is enclosed in densely packed, crosslinked, subpellicular microtubules closely underlying the plasma membrane. We isolated the subpellicular microtubules from bloodstream *Trypanosoma brucei (T. brucei)* parasites by use of a zwitterion detergent. These cold stable structures were solubilized by a high ionic strength salt solution and the soluble proteins which contained tubulin along with several other proteins were further fractionated by a Mono S cation exchange column, or by a tubulin affinity column. Two proteins were isolated, p52 (52kDa) from the Mono S column and p15 (15kDa) from the tubulin affinity column. Both proteins had the capacity to bind calf brain tubulin specifically and to polymerize calf brain tubulin into parallel closely packed microtubules. Under the same conditions, but without trypanosome-derived proteins, brain tubulin polymerized to single microtubules. It is thus suggested that the unique structural organization of the subpellicular microtubules is dictated by specific parasite proteins and is not an inherent property of the polymerizing tubulin. The *in vitro* reconstituted microtubule bundles are strikingly similar to the subpellicular microtubule network of the parasite.

When the solubilized subpellicular microtubules were fractionated on a cation exchange phosphocellulose column, p52 was eluted along with two other proteins of 41kDa and 36kDa. These proteins were found to be the glycosomal enzymes aldolase (41kDa) and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, 36kDa) by enzyme activity, antibody crossreaction and N-terminal sequencing. These enzymes were coprecipitated with tubulin in the presence of taxol, and aldolase had the capacity to polymerize tubulin and crosslink microtubules. Immunolocalization of anti aldolase and anti GAPDH antibodies did not show an interaction between these enzymes and the subpellicular microtubules. The question whether the copurification of aldolase and the subpellicular microtubules could reflect a physiological phenomenon or may be an experimental artifact is being discussed.

We used the phosphocellulose eluted proteins (p52, aldolase and GAPDH) for vaccination of rodents and checked their ability to protect animals against a *T. brucei* infection.

Vaccination of rodents using these proteins induced 100% protection against challenge with *T. brucei* (followed for 60 days after trypanosome challenge), whereas all control, non-vaccinated animals died within 7-12 days. The vaccinated animals showed a high titer of antibodies against the injected antigen which peaked after the third injection and remained high for the whole observation period.

The antibodies induced against the *T. brucei* antigen crossreacted with the antigen similarly isolated from *T. rhodesiense* and *T. evansi*, implying the possibility for developing a common vaccine against all trypanosome species.

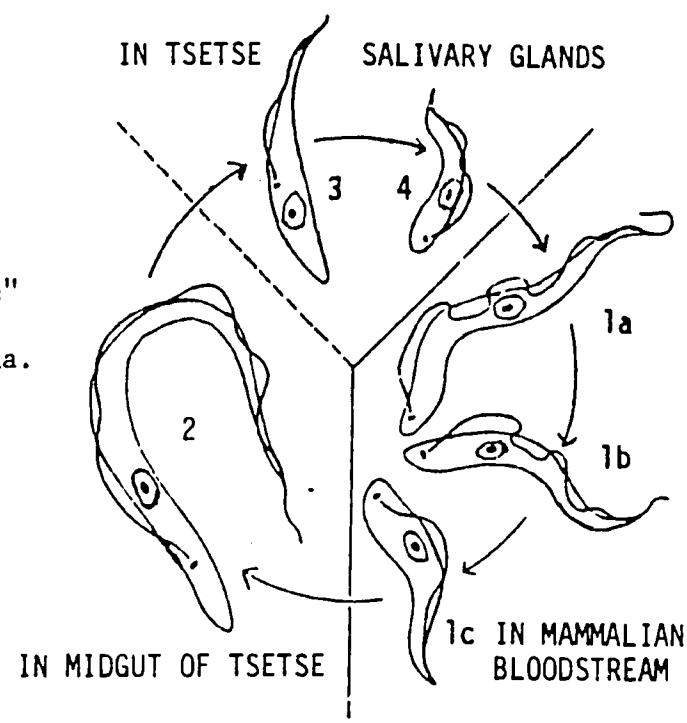
Trials to repeat this experiment were unsuccessful. However, sera of vaccinated and protected animals caused the agglutination of parasites *in vitro*, and immunolocalization of the agglutinated parasites showed no extracellular binding of antibody, but rather antibodies were detected within the parasites.

GENERAL INTRODUCTION

African trypanosomes (order Kinetoplastida; family Trypanosomatidae; genus Trypanosoma) are flagellated parasitic protozoa, causing sleeping sickness in humans and nagana in animals. Trypanosomiasis (African sleeping sickness and nagana) occurs in large areas of Africa, affecting about one million people in central and east Africa, with 20,000 new cases appear each year. In addition to presenting an enormous public health problem, trypanosomiasis is also a disease of cattle, having a tremendous social and economic impact since it prevents livestock from living in much of Africa, thus contributing to severe human malnutrition (Kolata, 1984; cf. Molyneux & Ashford, 1983).

The parasites are ingested by the tsetse fly where they undergo a cycle of development. All trypanosome species end their first phase of development as metacyclic forms in the fly mouth parts and salivary glands. These trypanosomes are then transmitted by the fly into the animals skin, then invade the local lymph vessels and move into the bloodstream where they undergo further development (see diagram below). Some parasites may then invade the connective tissue and in later stages the central nervous system (cf. Molyneux & Ashford, 1983).

Diagram of life cycle of trypanosomes of the *T. brucei* group. 1. Polymorphic forms in mammalian blood stream (1a "slender", 1b "intermediate" 1c "stumpy"). 2. Elongated form in fly midgut. 3. Crithidia. 4. Metacyclic trypanosome in salivary gland of tsetse fly.



Microtubules are found in all eukaryotic cells, and are relatively abundant in the Trypanosomatidae. Microtubules are cytoskeletal structures, known to be essential for various cellular structures, including cytoplasmic network, mitotic spindle, cillia and flagellar axonemas and neural processes. Functionally microtubules have a role in the development of cell shape, chromosome movement, cell migration, intracellular transport and secretion (cf. Dustin, 1984).

Microtubules are hollow cylindrical filaments of 20-25nm diameter. The major component of each filament is tubulin, which is a heterodimer composed of α and β subunits, having a molecular mass of 50-55kDa each. Microtubules purified from a variety of sources have been shown to consist of both tubulin and of a diverse group of microtubule associated proteins (MAPs) which copurified along with tubulin. The MAPs have been implicated in the regulation of microtubule assembly, by dramatically promoting the assembly of tubulin into microtubules, and in mediating the interaction of microtubules with other structural elements of the cell (cf. Littauer & Ginzburg, 1985; Olmsted, 1986).

All members of the Trypanosomatidae contain at least three structurally distinct classes of microtubules which are functionally different and which also differ greatly in their biochemical stability upon cell fractionation: the highly temperature labile microtubules of the mitotic apparatus (Solari, 1980), the salt and cold stable microtubules of the flagellar microtubules and the cold stable subpellicular microtubules. The high degree of stability of the microtubular cytoskeleton of the trypanosomes is in great contrast to those of many eukaryotic cells, where microtubules are often extremely labile and depolymerize easily during extraction. Various tubulin isotypes or posttranslation modifications of tubulin have been described, and it was implied that these variations were responsible for differential localization and stability of the trypanosome microtubules (Gull *et al.*, 1986; Gallo & Precigout, 1988; Schneider *et al.*, 1987; Sherwin *et al.*, 1987; Gallo *et al.*, 1988). However,

when total trypanosomal tubulin was isolated and characterized (Steiger *et al.*, 1984), it was found to be biochemically rather similar to mammalian brain tubulin and to exhibit *in vitro* polymerization characteristics which are similar to those of mammalian tubulin (Dolan *et al.*, 1986).

The analysis of trypanosomal α tubulin has shown that like in mammalian cells (Khawaja *et al.*, 1988; Kilmartin *et al.*, 1982; Kumar & Flavin, 1981; Schroder *et al*, 1985), this protein can be modified posttranslationally by a reversible detyrosination and retyrosination reaction (Steiger *et al.*, 1984). This reaction is highly specific for α tubulin, though the trypanosomal β tubulin also carries a tyrosine residue (unlike in mammalian cells (Wehland & Weber, 1987)) at its C-terminus. A more detailed study has demonstrated that stable microtubules, such as those of the subpellicular and the flagellar microtubules, contain detyrosinated α tubulin, while the newly generated microtubules contain much α tubulin with its C-terminal tyrosine still in place (Sherwin *et al.*, 1987 (in trypanosomes), Webster *et al.*, 1987 (in mammalian cells)). A second type of posttranslational modification is the acetylation of α tubulin (Piperno & Fuller, 1985; Piperno *et al.*, 1987; Maruta *et al.*, 1986 (in mammalian cells), and Schneider *et al.*, 1987 (in trypanosomes)). In the trypanosome, acetylated α tubulin is predominantly formed in stable microtubules (subpellicular and flagellar microtubules). Hence, neither tyrosination/detyrosination nor acetylation of α tubulin represent organelle-specific or function-related modifications. This view is further strengthened by the observation that the same tubulin isotypes (although at different stoichiometries) are found in the microtubules of the mitotic apparatus, the subpellicular and the flagellar microtubules (Schneider *et al.*, 1987; Dolan *et al.*, 1986; Russell *et al.*, 1984; Russell & Gull, 1984a). A very similar intracellular distribution of acetylated α tubulin has also been described in *T. cruzi* (Souto-Padron *et al.*, 1984).

Monoclonal antibodies against tubulin have been produced which selectively recognize subpopulations of the trypanosomal microtubules and rat brain tubulin (Gallo *et al.*, 1988; Gallo *et al.*, 1987; Gallo & Anderton, 1983). These results suggest that within the microtubule array of the subpellicular microtubules, some of the microtubules are distinct from all their neighbors. It is not clear at present if these specific epitopes represent posttranslational modifications or genetically different tubulin isotypes. A quartet of microtubules within the subpellicular microtubules has been identified which differs from its neighboring microtubules both structurally (Vickerman & Preston, 1976) and biochemically (Seebeck & Gehr, 1983). This quartet may be involved in the formation of the desmosome-like attachment structures of the flagellum to the cell body since it is always located underneath the area of contact between the flagellum and the cell body. The microtubules in this quartet may be the ones specifically recognized by the monoclonal antibodies.

The subpellicular microtubules contain a tight array of microtubules in close and stable contact with each other and with the cell membrane, forming a cage-like structure enclosing the cell body (cf. Molyneux & Ashford 1983; cf. De Souza, 1984; Russell & Dubremetz, 1986). The supramolecular arrangement of the subpellicular microtubules is unique to hemoflagellates and consists of uniformly spaced microtubules (about 50nm apart) held together by extensive crosslinking of adjacent microtubules. Interconnecting structures have been revealed using electron microscopy in a number of parasites (Souto-Padron *et al.*, 1984; Attias & De Souza, 1985; Jensen & Smaill, 1986, Aamodt & Culloti, 1986; Aamodt *et al.*, 1989; Bramblett *et al.*, 1987).

A tight attachment of the microtubular array to the cell membrane is suggested not only by their close apposition, but also by the stability of the membrane-microtubule complex upon cell fractionation (Dolan *et al.*, 1986; Bordier *et al.*, 1982; Voorheis *et al.*, 1979).

Mammalian microtubules usually appear as individual polymers, which run at a fixed distance of each other and occasionally form microtubule bundles. However, a close association of individual microtubules, forming a microtubular sheet, has not been demonstrated in mammalian cells. Since no unique tubulin isotype or a specific posttranslational modification of tubulin has been found in trypanosomes which could account for the special architecture of the subpellicular microtubules, the unique organization and stability of the subpellicular microtubules is most likely due to unique trypanosomal MAPs, which crosslink individual tubulin polymers and link the microtubule network to the plasma membrane.

Not much is known about the proteins participating in the construction of the subpellicular microtubules. Adligin, a 32kDa protein participating in the construction of a tight microtubule network in *Caenorhabditis elegans* (Aamodt and Culotti, 1986; Aamodt *et al.*, 1989) has been described, which was taxol precipitated and was able to crosslink microtubules *in vitro*. Antibodies directed against adligin crossreacted with a fibrous network in the cytoplasm of most cells of *C. elegans*, and crossreacted very well with neurons. It has been suggested that this protein may be bound to the interphase microtubules. COP proteins of 66, 41 and 35kDa have been isolated from the Trypanosomatidae *Crithidia fasciculata* cytoplasmic and subpellicular microtubules (Bramblett *et al.*, 1987) which can crosslink microtubules *in vitro*. However, one of these proteins has been found to be the glycosomal enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Bramblett *et al.*, 1989). MARP, a high molecular weight MAP2 like protein (Schneider *et al.*, 1988b) has been isolated from *T. brucei* subpellicular microtubules, which has a highly repetitive structure, and antibodies directed against it crossreacted weakly with hog brain MAP2. A 41kDa microtubule binding protein which may contribute to the interaction of the subpellicular microtubules and the plasma membrane in *T. brucei* has been described (Schneider *et al.*, 1988a). This protein was found to bind subpellicular microtubules in a calcium dependent manner

and was also found to contain covalently bound fatty acids. Since it was found to be in a low molar ratio to tubulin, it has been suggested that it might be localized at a particular site of the subpellicular microtubules.

The studies to be reported were aimed at the isolation and characterization of the proteins involved in the supramolecular organization of the subpellicular microtubules of *T. brucei*. The purification and characterization of these proteins is described in chapter I of this report.

During our study we observed that glycosomal enzymes can copurify with the subpellicular microtubules, giving rise to misleading results in the identification of unique trypanosomal MAPs. The nature of glycosomal enzymes and the question whether the association between glycosomal enzymes and the subpellicular microtubules could reflect a physiological phenomenon or may be an experimental artifact is specifically discussed in chapter II of this report.

The trypanosomal microtubules are an attractive target site for chemotherapeutic or immunological attack because of their possible involvement in controlling the life cycle of the parasite and possibly in controlling membrane-located physiological processes that are essential for the survival of the parasite in the host (Baum *et al.*, 1981). The use of microtubule proteins and proteins associated with the microtubules as possible vaccines against trypanosomiasis is described in chapter III of this report.

MATERIALS AND METHODS

Materials

Triton X-100 was obtained from Pierce Chemical Company, USA. Hoe-T-1566-1 was obtained from Hoechst Biochemical Company, West Germany. Zwittergent 3-14 was obtained from Calbiochem-Behring Corp., USA. Aprotinin (Trasylol), rabbit aldolase, rabbit GAPDH and yeast PGK were obtained from Boehringer Mannheim GmbH, West Germany. Phosphocellulose (P11) and DEAE cellulose (DE-52) were obtained from Whatman, U.K. Mono S column and Sephadex G-15 were obtained from Pharmacia, Sweden. Taxol was obtained from the National Cancer Institute, USA. Polyclonal anti tubulin antibodies and peroxidase conjugated anti rat IgG were obtained from BioMakor, Israel. Peroxidase conjugated anti mouse and anti rabbit IgG, and monoclonal anti β tubulin were obtained from Amersham, England. Gold conjugated anti rabbit, anti mouse and anti rat IgG were obtained from Janssen, Belgium. Centricon 10 and 30 were obtained from Amicon, USA. Affi Gel 10 was obtained from Bio-Rad, USA. Nitrocellulose membranes were obtained from Schleicher and Schuell, West Germany. All other chemicals were obtained from Sigma Chemical Company, USA.

Isolation of trypanosomes

The bloodstream form of *T. brucei* (subgroup formerly EATRO 1443, now KETRI 2693) maintained as a stabilate, was inoculated intraperitoneally in male SPD rats. Animals receiving $2x10^8$ cells each, usually showed a peak of parasitaemia at 10^9 cells/ml blood after 2 days. They were then anesthetized with ether and their blood was collected by cardiac puncture. Trypanosomes were separated from blood cells by passage through a DEAE-cellulose column. The parasites were eluted with ice cold phosphate buffered saline containing glucose (pH 8.0) as described by Lanham (1968). The collected trypanosomes were centrifuged at 3,000g for 15 min.

Preparation of detergent-resistant microtubules

T. brucei parasites were suspended in buffer A (10^{10} parasites per 16ml of buffer) consisting of 100mM PIPES (pH 6.9), 1mM EGTA, 0.1mM EDTA, 0.5mM $MgCl_2$ and 1mM dithiothreitol, supplemented with protease inhibitors (phenylmethylsulphonyl fluoride (1mM), benzamidine (10mM), aprotinin (10 units/ml) and leupeptin (10 μg/ml)), and with DNase (100 μg/ml) and RNase (100 μg/ml). A zwitterion detergent (Zwittergent 3-14) 10% in PBS was added to the parasite suspension to give a final concentration of 0.25%. After incubation for 30 min at 4°C the suspension was centrifuged at 10,000g for 20 min at 4°C. The pellet which contained flagellar and subpellicular microtubules was washed once with buffer A.

Solubilization and fractionation of microtubule preparations

The detergent insoluble fraction of *T. brucei* (4 mg protein/ml) was suspended in buffer A containing 0.75M NaCl and incubated for 30 min at 4°C. The suspension was then centrifuged at 100,000g for 30 min at 4°C. The supernatant was dialyzed overnight against buffer B (25mM PIPES, 1mM EGTA, 0.1mM EDTA, 0.5mM $MgCl_2$ and 1mM dithiothreitol). The dialyzed material was centrifuged again at 100,000g for 30 min (4°C) and both supernatant and pellet collected. The supernatant was loaded on a Mono S cation exchange column pre-equilibrated with buffer B, or on a phosphocellulose cation exchange column (12 mg protein per a 6 ml column), (as described by Williams & Detrich, 1979; Fellous *et al.*, 1977) pre-equilibrated with buffer B. Proteins were eluted from the columns by a salt gradient of 0-1M NaCl.

Affinity purification of 15 kDa microtubule associated protein

Solubilized subpellicular microtubules (in buffer A containing 0.75M NaCl) were run through a 30kDa cutoff membrane (Centricon 30), and the flow through was collected and concentrated on a membrane with a cutoff of 10kDa (Centricon 10). This material was then dialyzed against buffer A.

Phosphocellulose purified calf brain tubulin in MEM buffer (100 mM MES pH 6.5, 1mM EGTA, 0.5mM $MgCl_2$, 1mM dithiothreitol) (Fellous *et al.*, 1977) was coupled to affinity gel (Affi Gel 10) by overnight incubation at 4°C. The column was washed in the same buffer, and remaining active groups were blocked by incubating the resin with 1M ethanolamine HCl, pH 8.0, for 1 hr at 4°C. The column was equilibrated in buffer A and the dialyzed 10-30 kDa material was added and incubated with the resin for 1 hr at 37°C in the presence of 1mM GTP. The column was washed with buffer A and the tubulin binding proteins were batch eluted by washing the column with buffer A containing 0.75M NaCl. For polymerization experiments and liposome binding experiments the eluted material was dialyzed against buffer A.

Tubulin binding assay (tubulin overlay)

Tubulin binding assay was done as described by Kirsch *et al.*, 1989. Proteins were electrophoretically separated (Laemmli 1970) and transferred onto nitrocellulose membranes (Towbin *et al.*, 1979). For dot blot analysis 2μg protein was directly applied on the nitrocellulose membranes. The membranes were blocked with 5% fat-free milk in PBS for 30 min and were then equilibrated in PIPES buffer (100mM PIPES pH 6.9, 1mM EGTA, 0.5mM $MgCl_2$, 1mM dithiothreitol and 1mM GTP) for 2 min. The membranes were then incubated either in PIPES buffer (as a control) or in buffer containing 6.8μg/ml phosphocellulose purified bovine brain tubulin, and incubated for 2 hrs at 37°C. Taxol (20μM) was

added and the incubation was continued for another 30 min. Unbound tubulin was removed by three washes in PBS, 5 min each wash. Mouse anti β tubulin (diluted 1:1000 in PBS) was applied to both membranes and incubated for 1 hr at 37°C. The membranes were washed in PBS and the antigen-antibody complex was detected by further incubating the membranes with peroxidase conjugsted anti-mouse IgG for 1hr at 37°C and then washed in PBS as above. Blots were developed with 4-chloronaphthol.

Polymerization studies

Calf brain microtubule proteins were prepared by two cycles of polymerization, and pure tubulin was prepared from it by chromatography on a phosphocellulose column as described (Williams & Detrich, 1979; Fellous *et al.*, 1977). The purified tubulin or whole calf brain microtubules were incubated (30 min at 37°C) with 20μM taxol and 2.5mM GTP with or without the trypanosome purified proteins. The ratio of tubulin and the purified proteins was 6:1 (1.8 mg/ml of tubulin to 0.3 mg purified proteins/ml). Preformed microtubules were prepared by first incubating purified tubulin or whole calf brain microtubules in the presence of GTP and taxol as described above, and then further incubated in the presence of trypanosome purified proteins (and supplements of taxol and GTP to the above final concentration) for additional 30 min. The samples were negatively stained and observed under the electron microscope as described below.

Electron microscopy

For negative staining a drop of the mixture was put on a 300 mesh formvar coated grid, or a 1:20 dilution on 300 mesh carbon coated grids. After 30 sec the samples were negatively stained for 10 sec with 1% uranyl acetate (in water). Grids were examined at 80 kV in a Phillips EM 410 microscope.

For thin section micrographs the samples were fixed with 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 45 min, washed and post fixed with 2% osmium tetroxide in 0.1M cacodylate buffer (1 hr) all at room temperature. The pellets were washed and were then stepwise dehydrated in ethanol and embedded in Poly/Bed (Hayat, 1972). Sections were examined in a Phillips EM 410 microscope.

Antibodies

Polyclonal antibodies directed against p15 were raised in rabbits which were injected 3 times subcutaneously with the antigen. Injections were made 2 weeks apart. Silver stained gel pieces of SDS-PAGE containing p15 were used as antigens for injection, about 5μg of p15 were used for every injection. The first injection contained complete Freund's adjuvant while the second and third injections contained incomplete Freund's adjuvant.

Polyclonal antibodies directed against p52 were raised in BALB/c mice and in rabbits, with the same injection scedual as described for p15. Antigen used for injection was Mono S purified p52 which was run on SDS-PAGE. Gel pieces containing the protein were excised and injected.

Polyclonal antibodies directed against aldolase were a generous gift of C. Clayton, and monoclonal antibodies directed against GAPDH were a generous gift of F. Opperdoes. Monoclonal anti tubulin antibodies were commercially available from Amersham. Polyclonal anti tubulin antibodies were commercially available from BioMakor.

Western blot analysis

Proteins were separated on SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). The membranes were blocked with 5% fat-free milk in PBS for 30 min and were then incubated in the presence of anti-

bodies for 1 hr at 22°C. The membranes were washed in PBS containing 0.5% Tween 20, 3 times 10 min each, and were then incubated for 1 hr at 22°C with peroxidase conjugated anti rabbit, anti mouse or anti rat antibodies. The membranes were washed as above, and the bound antibody was detected using 4-chloronaphthol.

Copolymerization of p15 or p52 with tubulin: Immunogold labeling

A drop of the preformed polymers (prepared as described above) was applied to a formvar coated copper grid (as described above), fixed for 10 min in 0.1% glutaraldehyde, washed 3 times in PBS, 2 min each wash, and incubated overnight at 4°C with anti p15 or anti p52 antibody (diluted 1:50 in PBS) or with commercially available monoclonal anti tubulin antibody (Amersham) (diluted 1:100 in PBS). The grids were then washed in PBS and incubated for 30 min at 22°C in 10nm gold conjugated anti rabbit IgG (for anti p15 antibodies) or anti mouse IgG (for anti tubulin and anti p52 antibodies). The grids were washed again in PBS and were then negatively stained (as described above) and were observed under the Philips EM 410 microscope.

Immunolocalization

T. brucei were fixed for 1 hr at 22°C in 0.1% glutaraldehyde and cryosectioned (Tokuyasu, 1973; Griffiths *et al.*, 1984). Sections were collected on 200-mesh carbon over formvar-coated copper grids, and quenched for 10 min at 22°C in PBS containing 0.02M glycine, 1% BSA, 2% gelatin and 0.05% Tween 20. The grids were then incubated overnight at 4°C with the antibody. The grids were washed 5 times in PBS, 2 min each wash, and incubated for 30 min at 22°C with 10nm gold conjugated anti mouse, anti rabbit or anti rat IgG. The grids were washed again in PBS, then in water and were then stained in 1% uranyl acetate (pH 7.2) for 10 min at 22°C. The grids were washed in water and embedded for 10 min in methyl cellulose containing 0.1% uranyl acetate. Double labeling was

performed by incubating the grids overnight at 4°C with the first anti sera, washing the grids in PBS as above and bound antibody was detected by further incubating the grids for 1 hr at 22°C with 15nm gold conjugated anti mouse or anti rabbit IgG. The grids were washed again in PBS and incubated for 1 hr at 22°C with the second anti sera, washed in PBS and bound antibody was detected using 5nm gold conjugated anti mouse or anti rabbit antibodies. The grids were washed in PBS, stained in uranyl acetate and embedded in methyl cellulose as described above. Grids were examined at 80 kV in a Phillips EM 410 microscope.

Liposome binding assay

Unilammelar liposomes were prepared by solubilizing soybean asolectin in methanol:acetone 1:1 (v/v), evaporating it and resuspending the lipids as a 20mg/ml suspension in 10mM Hepes and 100mM sucrose, pH 7.2. The suspension was then sonicated in a bath sonicator for 30 min and liposomes were purified by gel filtration through Sepadex G-15. 10μl of the liposome suspension were mixed with 1μg p15 or p52, incubated at 22°C for 10 min and centrifuged for 20 min at 100,000g. Both pellet and supernatant were collected, run on SDS-PAGE and silver stained.

Binding of microtubules to liposomes

Microtubules (polymerized as described above) were mixed (1:1 v/v) with asolectin liposomes (prepared as described above). A drop of the mixture was applied to a formvar coated copper grid at 1 min and 10 min after mixing and the grids were negatively stained as described above.

ELISA experiments

Wells of microtiter plates were coated with 50μl of 50μg/ml phosphocellulose purified proteins and incubated overnight at 4°C. Unbound antigen was removed and the plates were blocked for 1 hr at 22°C in PBS containing 1% BSA. Antiserum (diluted 1:100 in PBS) was added and incubated for 2 hrs at 4°C. The wells were washed in PBS (3 times, 2 min each wash), and peroxidase conjugated anti rabbit or anti rat antibodies were applied and incubated for 1 hr at 37°C. The wells were then washed in PBS as above and bound antibody was detected using ABTS as a substrate.

V8 digestion

V8 (10μg/ml) was applied to 2μg PGK or p52, incubated for 30 min at 22°C and proteins were then separated on SDS-PAGE and silver stained.

Electroelution

The phosphocellulose purified proteins were separated on SDS-PAGE, stained in coomassie and the appropriate bands were excised. The gel pieces were put in an ISCO electrophoretic sample concentrator and electroelution was carried out for 2 hrs at 50mA in buffer containing 0.19M glycine, 25mM Tris, 0.1% SDS and 14mM thioglycolate.

N-terminal sequence analysis

N-terminal sequence analysis of the electroeluted proteins was done according to Hunkapiller *et al.*, (1983), using a pulsed liquid gas phase protein microsequenser (model 475A Applied Biosystems Inc. CA).

Aldolase activity

Aldolase activity was measured using an Aldolase colorimetric kit (Sigma).

PGK activity

PGK activity was measured according to the method described by M. Parsons (personal communications). 300μl Buffer A (100mM Tris-HCl pH 7.5, 1mM EDTA, 5mM MgSO4, 1mM dithiothreitol, 0.3mM NADH and 1mM ATP) were mixed with 150μl water, 50μl GAPDH (50μg/ml), 50μl 5.6mM 3-phosphoglycerate and 50μl of the sample. The reaction was monitored immediately at 340nm in a Zeiss spectrophotometer.

Taxol precipitation

Purified calf brain tubulin (1.8 mg/ml) was incubated in the presence of the solubilized (and dialyzed) subpellicular microtubules (0.8 mg/ml), 20μM taxol and 2.5mM GTP at 37°C for 30 min and centrifuged at 45,000g for 20 min at 20°C. The precipitated pellet was collected and run on SDS-PAGE.

Vaccination experiments

The proteins eluted from the phosphocellulose column were injected into Wistar male rats and Swiss male mice at various time schemes. The protein (25μg) was injected subcutaneously and into the footpad. The subcutaneous injections included complete Freund's adjuvant (CFA) on first injection and incomplete Freund's adjuvant on second and third injections. Control animals were injected once with CFA. Both vaccinated and control animals were challenged with 500 parasites (which were previously isolated as described above).

Aggregation experiments,

$2x10^9$ parasites/ml (isolated as described above) were mixed 1:1 (v/v) with the sera, and a drop of the mixture was put on a glass slide and observed under a light microscope.

Internalization of antibodies

The parasites were mixed with the sera (or with PSG as a control) as described above and incubated for 1 min and 15 min at 22°C. The parasites were then centrifuged for 5 min at 3000g at 22°C, excess serum was removed and the parasites were fixed for 1 hr at 22°C in 0.1% glutaraldehyde. Fixed parasites were cryosectioned (Tokuyasu, 1973; Griffiths *et al.*, 1984), and sections were collected on 200-mesh carbon over formvar-coated copper grids. The grids were blocked for 10 min at 22°C in PBS containing 0.02M glycine, 1% BSA, 2% gelatin and 0.05% Tween 20. The grids were then incubated for 1 hr at 22°C with 10nm gold conjugated anti rat antibodies, washed 5 times in PBS, 2 min each wash, then in water and were stained in 1% uranyl acetate (pH 7.2) for 10 min at 22°C. The grids were washed in water and embedded for 10 min in methyl cellulose containing 0.1% uranyl acetate. Grids were examined at 80 kV in a Phillips EM 410 microscope.

Miscellaneous procedures

Protein concentration was determined using the Bio-Rad microassay described by Bradford (1976) with bovine serum albumin as standard. Densitometric scanning of stained slab gels were performed using a Bio-Rad 620 video densitometer. Silver staining of SDS-PAGE was done as described by Wray *et al.*, (1981). Amino acid analysis was performed according to Moore & Stein (1954), using cation exchange column followed by post-column ninhydrin detection.

I. Isolation of Subpellicular Microtubule Proteins from *Trypanosoma brucei* which Mediate Crosslinking of Microtubules and Crosslinking of Microtubules to Phospholipids

INTRODUCTION

The first attempts to identify proteins associated with microtubules parallels the earliest investigations of the biochemical constituents of well-defined microtubule arrays. The first detailed studies of one of these was performed by Gibbons (1965), who demonstrated that dynein could be selectively released and rebound to axonemes of *Tetrahymena*. The specific reassociation of proteins with the microtubule lattice is still one of the major criteria for identifying putative MAPs. The discovery by Weisenberg (1972) that microtubules can be assembled *in vitro* from extracts of brain tissue laid the foundation for the identification of protein components that associated with the microtubule lattice. By enriching for microtubule fractions through repetitive cycles of temperature-dependent assembly and disassembly (Shelanski *et al.*, 1973), proteins were identified that copurify in constant stoichiometry with tubulin and whose association with microtubules depends upon the formation of polymers. Fractions enriched in copurifying proteins were isolated from mammalian brain which promoted the assembly of microtubules at much lower concentrations than did tubulin alone and stabilized existing polymers (e.g. Murphy & Borisy, 1975), and high molecular weight MAPs were shown to form projections on the surface of the microtubules (e.g. Sloboda & Rosenbaum, 1979). Another class of proteins remains associated with cold-stable microtubules in brain microtubule preparation (Margolis & Rauch, 1981), which depolymerize in the presence of calmodulin and low calcium concentrations (Job *et al.*, 1982). The

discovery of taxol as a drug promoting the assembly of microtubules *in vivo* and *in vitro* (Horwitz *et al*, 1982), allowed the isolation of tubulin and its associated molecules from tissues containing low concentrations of microtubules.

The subpellicular microtubules of the trypanosome are cold stable, a property which poses difficulty on the purification of its MAPs. There have been reports on the purification of these microtubules by the use of Triton (Stieger *et al.*, 1984) or by solubilizing the microtubules with calcium (Dolan *et al.*, 1986). However these methods did not lead to pure subpellicular microtubule preparations. We have developed a method for selectively purifying the subpellicular microtubules and identified two subpellicular MAPs of 52kDa (p52) and of 15kDa (p15). In order to identify these proteins as MAPs, we used the accepted criteria for MAPs (cf. Olmsted, 1986), i.e. that they should copurify with tubulin, promote the polymerization of microtubules, and also that antibodies directed against them colocalize with anti tubulin antibodies. The specificity of these proteins as subpellicular MAPs was confirmed by their specific immunolocalization, and by their ability to crosslink microtubules to a structure similar to the original form of the subpellicular microtubules. Furthermore, these MAPs were found to bind phospholipid liposomes, and were therefore suggested to have a double role of both crosslinking the microtubules and in linking the microtubules to the plasma membrane, thereby contributing to the stable architecture observed in the subpellicular microtubules.

RESULTS

Isolation of trypanosomal microtubules

Four detergents were screened for their suitability for preparation of detergent resistant cytoskeletons from *T. brucei*. As judged by electron microscopy of thin sections of the preparations of the four (Triton X-100, CHAPS, Hoe-1566-1 and Zwittergent 3-14), Zwittergent 3-14 gave the most suitable preparations in terms of structural integrity and purification from cellular membranes. Fig. 1B shows that the subpellicular microtubule cages and the flagellar microtubules are the dominant structures found in the detergent insoluble preparations. The membranes surrounding the flagellar axoneme and the plasma membranes (Fig. 1A) were efficiently removed by the detergent. Fig. 1D shows a tangential cut through the trypanosome subpellicular array of microtubules (an enlargement of a section of Fig. 1B), revealing periodic crosslinks between individual microtubules (see arrow).

Forty percent of the proteins remained insoluble after Zwittergent 3-14 treatment. Densitometer scanning of stained slab gels revealed that the insoluble fraction was two-fold enriched with tubulin as compared with total cell protein (Fig. 2 lanes 1 and 2). The position of tubulin was determined using anti tubulin monoclonal antibodies (Fig. 3B).

Solubilization and partial purification of microtubule proteins

Detergent insoluble microtubules were solubilized by 0.75M NaCl, a condition known to solubilize calf brain microtubules (Vallee, 1982). About 50% of the insoluble proteins of cytoskeletal preparations were solubilized, and no further solubilization was achieved by repeated treatment of the insoluble residue with NaCl. The protein pattern of the salt insoluble residue is given in Fig. 2 lane 3. Electron microscopy of the remaining insoluble residue showed that it consisted by and large of intact flagellar microtubules with a paraxial

rod still intact and attached (Fig. 1C). Thus the solubilized proteins of *T. brucei* contain little or no components of the flagellar microtubules.

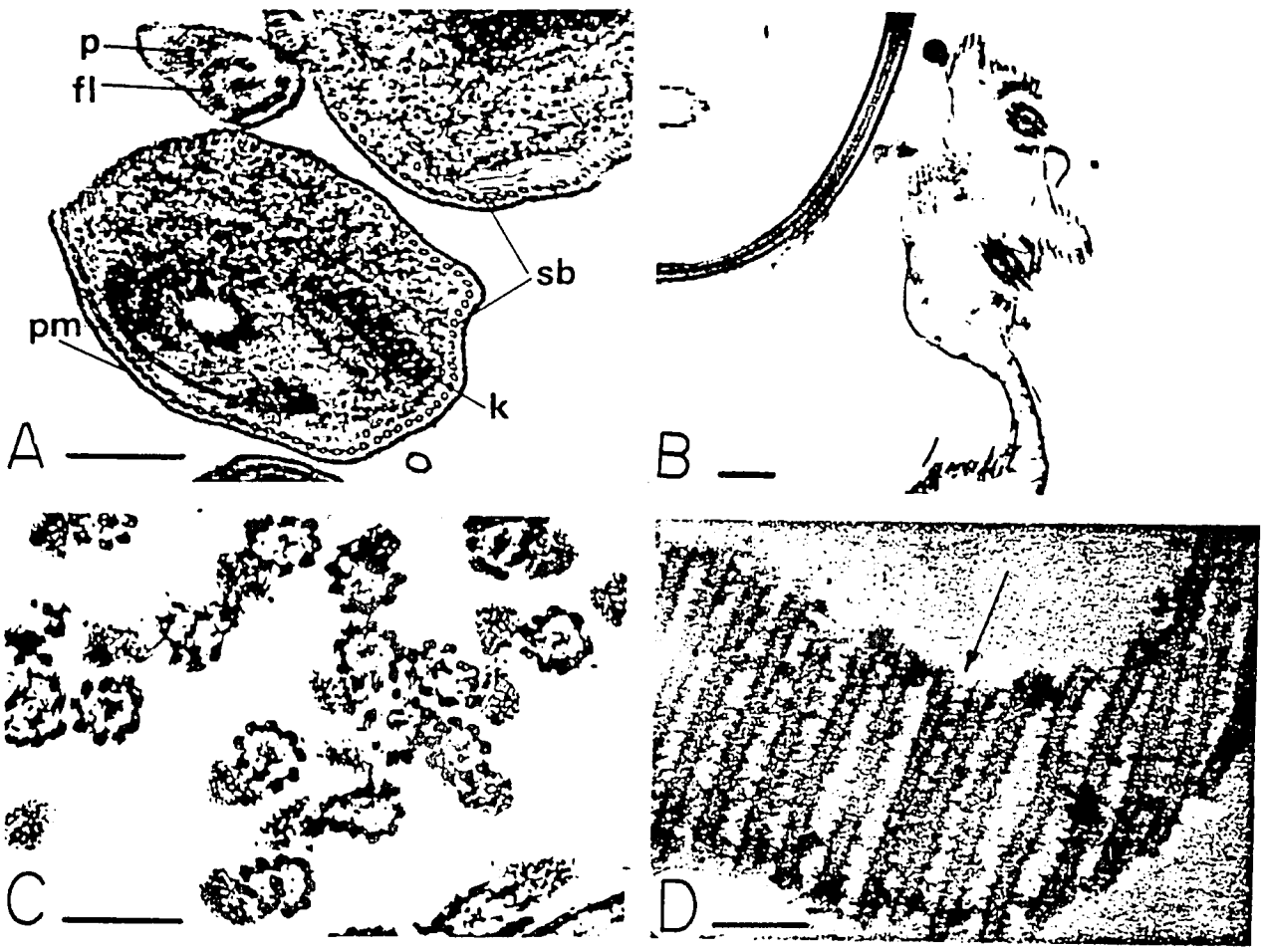


Figure 1. Electron micrographs of thin sections of *T. brucei* and detergent resistant cytoskeletal microtubules, embedded in Poly/Bed 812. (A) is a section of intact *T.brucei*. Note the regularly spaced subpellicular microtubules and the flagellar microtubules. sb-subpellicular microtubules, fl-flagellar microtubules, k-kinetoplast, pm-plasma membrane, p-paraxial rod. (B) represents the detergent (Zwittergent 3-14) resistant cytoskeleton. (A-B, bar, 400nm). (C) The detergent and salt resistant fraction. Flagellar microtubules with attached paraxial rods are the dominant structures in the residue. (bar, 300nm). (D) shows a high magnification (bar, 100nm) of a cut through the subpellicular microtubule network. Note the inter-microtubule crosslinks (arrow).

The soluble fraction was dialyzed extensively, in the course of which about 50% of the proteins precipitated (Fig. 2 lane 4). The fraction which remained in solution contained tubulin as well as several other proteins (Fig. 2 lane 5). Tubulin is not retained on phosphocellulose cation exchange columns (Fellous *et al.*, 1977). In order to separate between tubulin and its associated proteins, we fractionated the proteins that remained in solution (Fig. 2 lane 5) on phosphocellulose columns. Most of the soluble proteins including tubulin were not retained on the column (Fig. 2 lane 6), the retained proteins were eluted as one peak at around 0.5M NaCl and contained proteins of 52kDa, 41kDa and 36kDa (Fig. 2 lane 7).

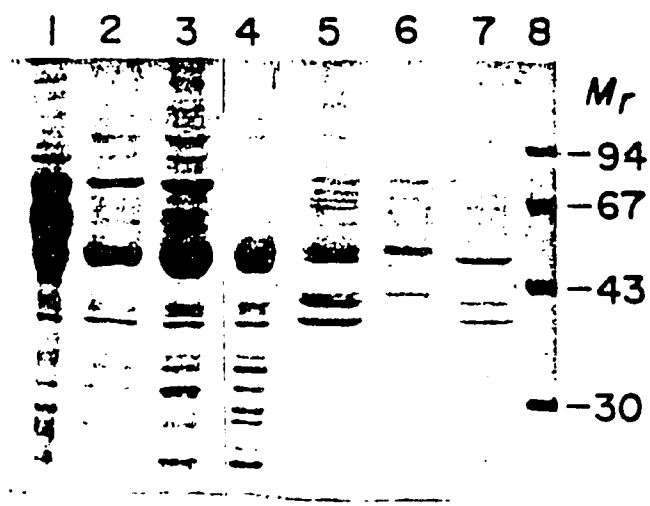


Figure 2. Steps in the isolation of *T. brucei* microtubule proteins - SDS 10% PAGE analysis. Lane 1 shows the spectrum of proteins in a *T. brucei* homogenate. Lane 2 shows the proteins of a detergent resistant microtubule preparation. Lane 3 represents the proteins which were not solubilized by 0.75M NaCl treatment. Lanes 4 and 5 represent the proteins solubilized by 0.75M NaCl. The precipitate that formed during dialysis is given in lane 4, and the proteins remaining in the soluble fraction are given in lane 5. Lane 6 represents proteins which were not retained by a phosphocellulose column which was loaded with the soluble proteins shown in lane 5. Lane 7 represents the proteins that were retained on the phosphocellulose column and eluted by salt. Lane 8 represents molecular weight markers, their molecular mass given in kilodaltons.

Tubulin binding proteins

To assess which of the proteins eluted from the phosphocellulose columns bind tubulin specifically, we used a tubulin binding assay, consisting of proteins blotted onto nitrocellulose paper and then overlayed with bovine brain tubulin. Bound tubulin was detected by means of monoclonal antibodies directed against tubulin (Fig. 3A). Control blots were incubated only with the antibody (Fig. 3B). Figure 3A (lanes 1-4) shows that total cell homogenate (lane 1), detergent insoluble proteins (lane 2), subpellicular microtubules solubilized by salt and precipitated during dialysis (lane 3) and the remaining soluble subpellicular microtubule proteins (lane 4) all bound tubulin and were also recognized by anti tubulin antibodies (Fig. 3B lanes 1-4). It is not surprising to see an enhancement in lanes 1-4 A as compared to 1-4 B since these preparations contain endogenous trypanosome tubulin which by itself binds calf brain tubulin. However, among the phosphocellulose eluted proteins (Fig. 3 lane 5) which do not contain endogenous trypanosome tubulin (Fig. 3B lane 5), one protein of about 52kDa bound exogenous tubulin specifically (Fig. 3A lane 5).

Fractionation on Mono S columns

Tubulin binding protein was purified using Mono S cation exchange columns. The column was loaded with salt solubilized (and dialyzed) subpellicular microtubule proteins (Fig. 4A lane 1). Most of the soluble proteins including tubulin were not retained on a Mono S column (Fig. 4A lane 2). The retained proteins were eluted as two distinct peaks at 0.15M and 0.19M NaCl (Fig. 4B), and contained proteins of molecular weights of 53kDa and 52kDa respectively (Fig. 4A lanes 3 and 4). In order to check which of the proteins is the tubulin binding one, each of the Mono S eluted proteins were dot blotted and exposed to the tubulin binding assay (Fig. 5). The 52kDa Mono S protein was found to bind tubulin specifically (Fig. 5A,C), and the 53kDa protein had no tubulin binding capacity (Fig. 5B,D).

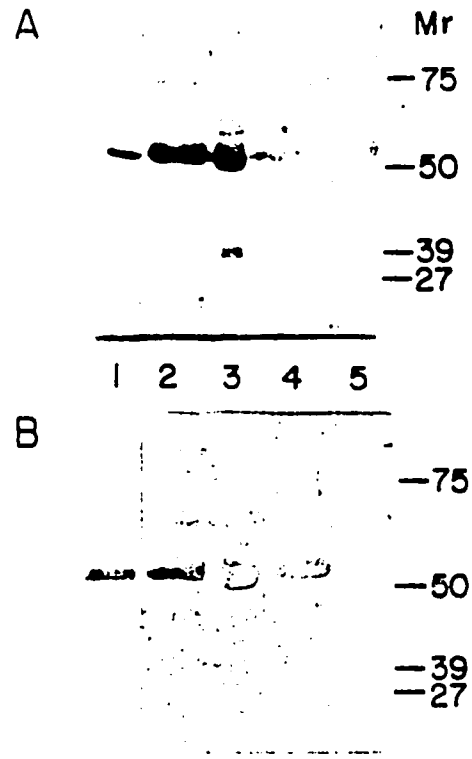


Figure 3. Tubulin binding assay. Proteins were blotted onto a nitrocellulose paper and exposed to calf brain tubulin (A) which was then detected by anti β tubulin antibodies. B represents the controls showing anti β tubulin binding without prior exposure to exogenous tubulin. The proteins that were run on SDS 10% PAGE and blotted, were: Lane 1 total cell homogenate (as in Fig. 2 lane 1). Lane 2 detergent resistant cytoskeletal proteins (as in Fig. 2 lane 2). Lane 3 proteins solubilized by salt and precipitated during dialysis (as in Fig. 2 lane 4). Lane 4 proteins which were solubilized by salt, remained soluble after dialysis and loaded on a phosphocellulose column (as in Fig. 2 lane 5). Lane 5 proteins eluted from a phosphocellulose column (as in Fig. 2 lane 7). On the right are given prestained Sigma molecular weight markers in kilodaltons.

Taxol precipitation

Taxol is an antimitotic drug known to promote microtubule assembly (Vallee, 1982). We added taxol (and GTP) to the solubilized subpellicular microtubules (Fig. 4A lane 1) in the presence of calf brain tubulin (Fig. 4A lane 5). The precipitate that formed was analyzed by gel electrophoresis and showed that proteins of 36kDa, 41kDa and 52-53kDa coprecipitated with tubulin (Fig. 4A lane 6).

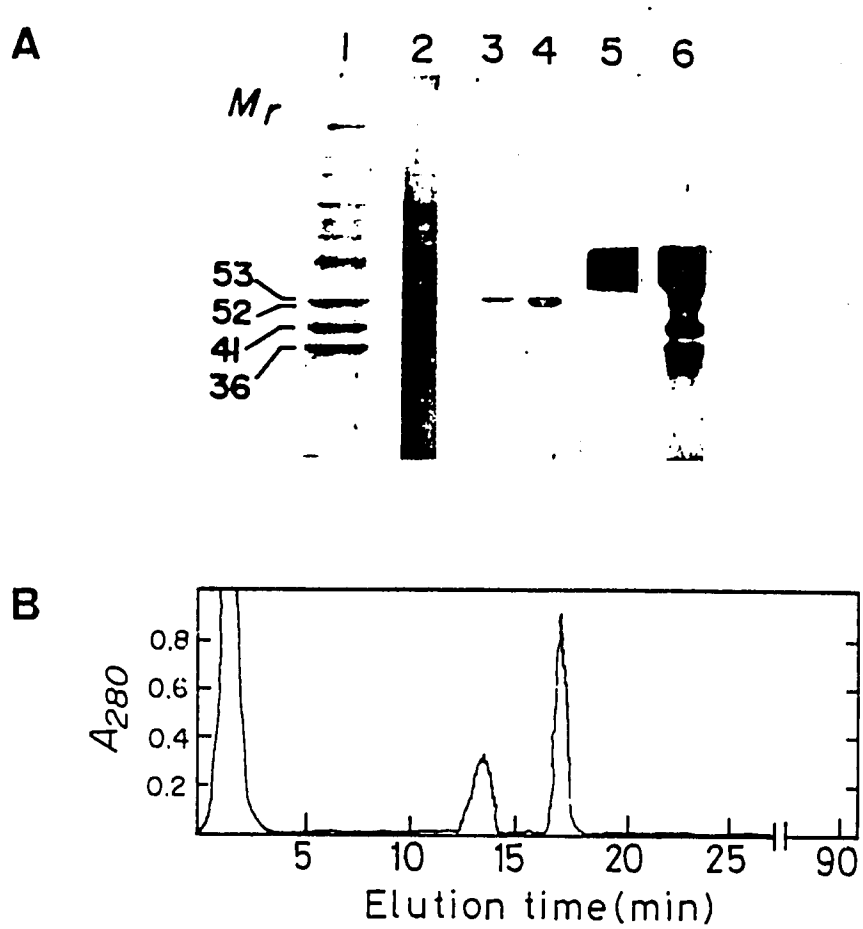


Figure 4. Fractionation of solubilized subpellicular microtubules on a Mono S cation exchange column. The proteins were eluted by a salt gradient of 0-1M NaCl, starting 4 min after application and continuing for 93 min. A. SDS 10% PAGE analysis, B. Elution pattern. NaCl solubilized proteins were dialyzed, the precipitate removed and the soluble fraction was loaded on the column (panel A, lane 1). The flow-through proteins (first peak in the elution profile (panel B)) are shown in panel A lane 2 and the proteins retained on the column and eluted at 0.15M (about 14 min) and 0.19M NaCl (about 17 min) gradient (see panel B) are given in panel A lanes 3 and 4, respectively. Lane 5 represents phosphocellulose purified calf brain tubulin. Lane 6 represents the proteins of the soluble fraction (given in lane 1) which coprecipitated with calf brain tubulin, in the presence of taxol and GTP. Major polypeptides are identified by their molecular mass in kilodaltons.

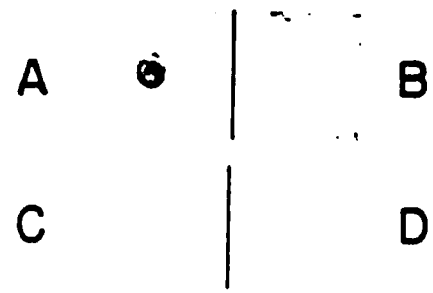


Figure 5. Tubulin binding to Mono S eluted 52kDa and 53kDa proteins: dot blot analysis. Proteins were applied onto the nitrocellulose membrane and incubated in the presence of tubulin, and bound tubulin was detected by anti tubulin antibodies (A,B). C and D were incubated only with anti tubulin antibodies without prior exposure to tubulin. The proteins that were applied onto the membrane were: A,C: 52kDa. B,D: 53kDa.

Trypanosome microtubules were separated on SDS-PAGE and electroblotted onto a nitrocellulose membrane (Fig. 6 lane 1). Western blot analysis showed that antibodies directed against a mixture of the Mono S eluted proteins recognized the two proteins of 52kDa and 53kDa (Fig. 6 lane 2). Antibodies directed against the 52kDa protein (raised either in rabbits or in mice) recognized this protein only (Fig. 6 lane 3). When these antibodies were reacted against the taxol precipitated proteins (Fig. 6 lane 4), only the the 52kDa protein was detected (Fig. 6 lanes 6 and 7), suggesting that only the 52kDa protein precipitated by taxol. The location of calf brain tubulin identified using monoclonal anti β tubulin antibodies is given in Fig. 6 lane 5. (polyclonal anti tubulin antibodies had the same labeling pattern (not shown). Of note is that antibodies directed against the 52kDa protein did not crossreact with calf brain microtubules in western blot experiments (not shown).

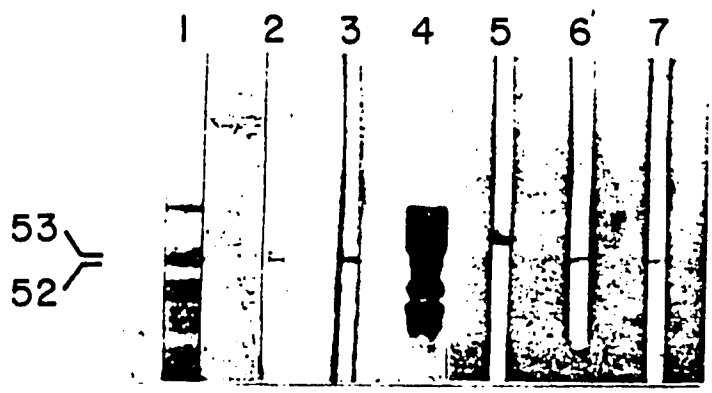


Figure 6. Western blot analysis. Detergent resistant cytoskeletal proteins (lane 1-3, as shown in lane 1) and taxol precipitated proteins (lane 4-7, as shown in lane 4), were blotted onto a nitrocellulose membrane and were exposed to the following antibodies: Lane 2 was exposed to antibodies directed against the 52kDa and 53kDa proteins eluted from the Mono S column. Lane 3 was exposed to antibodies directed against the 52kDa protein. Lane 5 was esposed to monoclonal antibodies directed against β tubulin. Lane 6 was exposed to antibodies directed against 52-53kDa, and lane 7 to antibodies directed against the 52kDa protein. Major polypeptides are identified by their molecular mass in kilodaltons.

Effect of Mono S purified trypanosome proteins on microtubule organization

Bovine brain tubulin incubated for 30 min at 37°C with 20μM taxol and 2.5mM GTP (conditions favorable for microtubule polymerization (Vallee, 1982)) formed long strands of single microtubules (Fig. 7A). The inset shows a higher magnification of the strands in which one can observe parallel lines, the fine structure of protofilaments as viewed in negative stained preparations.

Addition of the 52kDa protein purified from the Mono S column to calf brain tubulin at a ratio of 1:6 in the presence of taxol and GTP as above, led to the formation of microtubule bundles in which strands of microtubules were closely aligned (Fig. 7B). A higher ratio of the 52kDa protein to tubulin did not lead to a higher degree of bundling (a qualitative observation). At a larger magnification (Fig. 7C) one can identify microtubules connected by comb-like regular bridges. The bridges are about 7.2nm apart (center to center) corresponding roughly to one bridge per tubulin dimer. Bundles are rather similar to the bundles seen in a negatively stained preparation of frozen and thawed *T. brucei* (Fig. 7D and E). The fraction containing the 53kDa protein had no bundling capacity.

Of note is the fact that dissociated brain microtubule preparations which contain tubulin as well as endogenous microtubule associated proteins (MAPs) form single strands of microtubules in the presence of taxol and GTP. Without taxol no polymerization or bundling occurred in any of the experiments described above. Preformed microtubules consisting of purified tubulin were able to bundle after the addition of the 52kDa protein. However when microtubules were preformed from whole microtubules (containing tubulin and endogenous MAPs), addition of trypanosomal proteins did not cause any bundling (Fig. 7F), suggesting that the site of tubulin binding to the bundling protein was already occupied by the endogenous MAPs.

Figure 7. Microtubules polymerized *in vitro* with or without *T. brucei* proteins. (A) represents *in vitro* polymerized (in the presence of taxol and GTP) calf brain microtubules (bar, 200nm). Note the fine structure of the microtubules and their protofilaments in the inset (bar, 50nm). (B) represents a bundle of closely associated microtubules polymerized in the presence of phosphocellulose-retained *T. brucei* proteins (bar, 200nm). (C) is a higher magnification of (B) (bar, 100nm). Note the periodic crosslinks (an example indicated by the comb) that can be seen in the spaces between the microtubules. (D) shows a negative staining of a frozen and thawed parasite with its network of microtubules (bar, 300nm) which in a higher magnification (bar, 400nm) shows tight association between individual microtubules brought about by periodic crosslinks (E). (B) and (C) are strikingly similar to (E) which is the *in vivo* presentation. (F) represents calf brain microtubules prepolymerised (in the presence of taxol and GTP) and then incubated in the presence of the 52kDa protein for another 30 min in the presence of taxol and GTP (bar, 200nm).

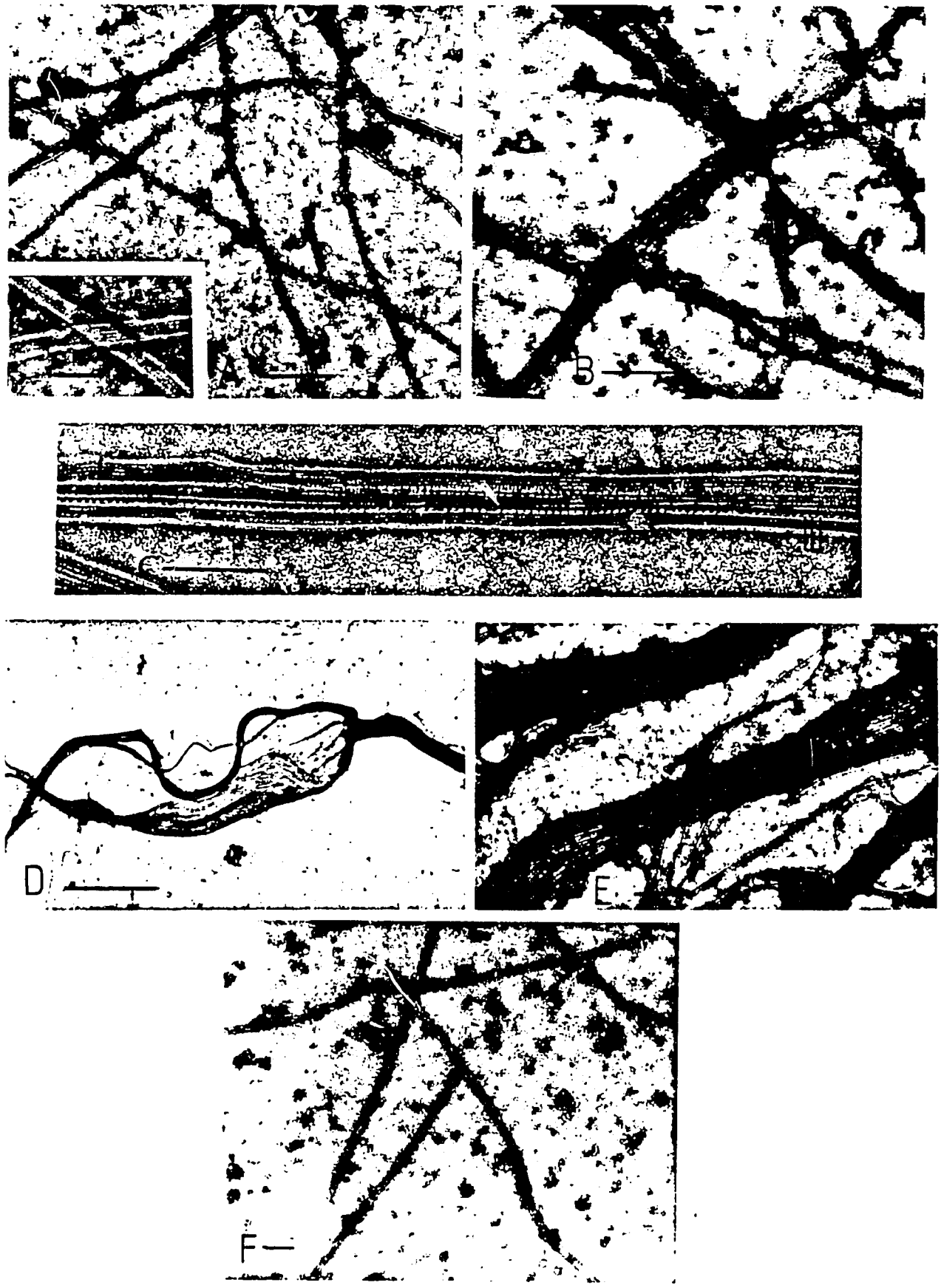


Figure 7

Copolymerization of the 52kDa protein (p52) with tubulin

Calf brain tubulin was polymerized in the presence of solubilized (and dialyzed) subpellicular microtubules, taxol and GTP, fixed and immunolabeled with antibodies directed against p52 (Fig. 8A and B) or with monoclonal anti tubulin antibodies (Fig. 8C). The similar binding pattern of the two antibodies suggests that p52 has the ability to copolymerize with tubulin.

Immunolocalization of p52

T. brucei were fixed, cryosectioned and immunolabeled with anti p52 antibodies (bound antibody was detected using 15nm gold conjugated anti rabbit antibodies), or were double labeled with anti p52 and anti tubulin antibodies (bound tubulin was detected using 5nm gold conjugated anti mouse antibodies). Labeling of the parasite with anti tubulin antibodies was necessary in order to locate the position of microtubules in the cell. Results show that p52 antibodies labeled the periphary of the cell (Fig. 9A and 9B), i.e. the subpellicular microtubules, while anti tubulin antibodies labeled also the cytoplasmic microtubules (Fig. 9B). These results confirm the colocalization of p52 with the subpellicular microtubules, and show that it is a specific subpellicular MAP.

Figure 8. Copolymerization and immunolocalization of the p52 antigen by immunogold labeling and negative staining. Solubilized subpellicular microtubules were polymerized in the presence of calf brain tubulin and taxol and GTP, a drop was put on a grid, the polymers were fixed in 0.1% glutaraldehyde and immunolabeled with a 1:50 dilution of polyclonal anti p52 antibodies (A,B, bar, 200nm) or with monoclonal anti tubulin antibodies (C, bar, 200nm). Bound antibodies were detected using anti mouse 10nm gold conjugated antibodies.

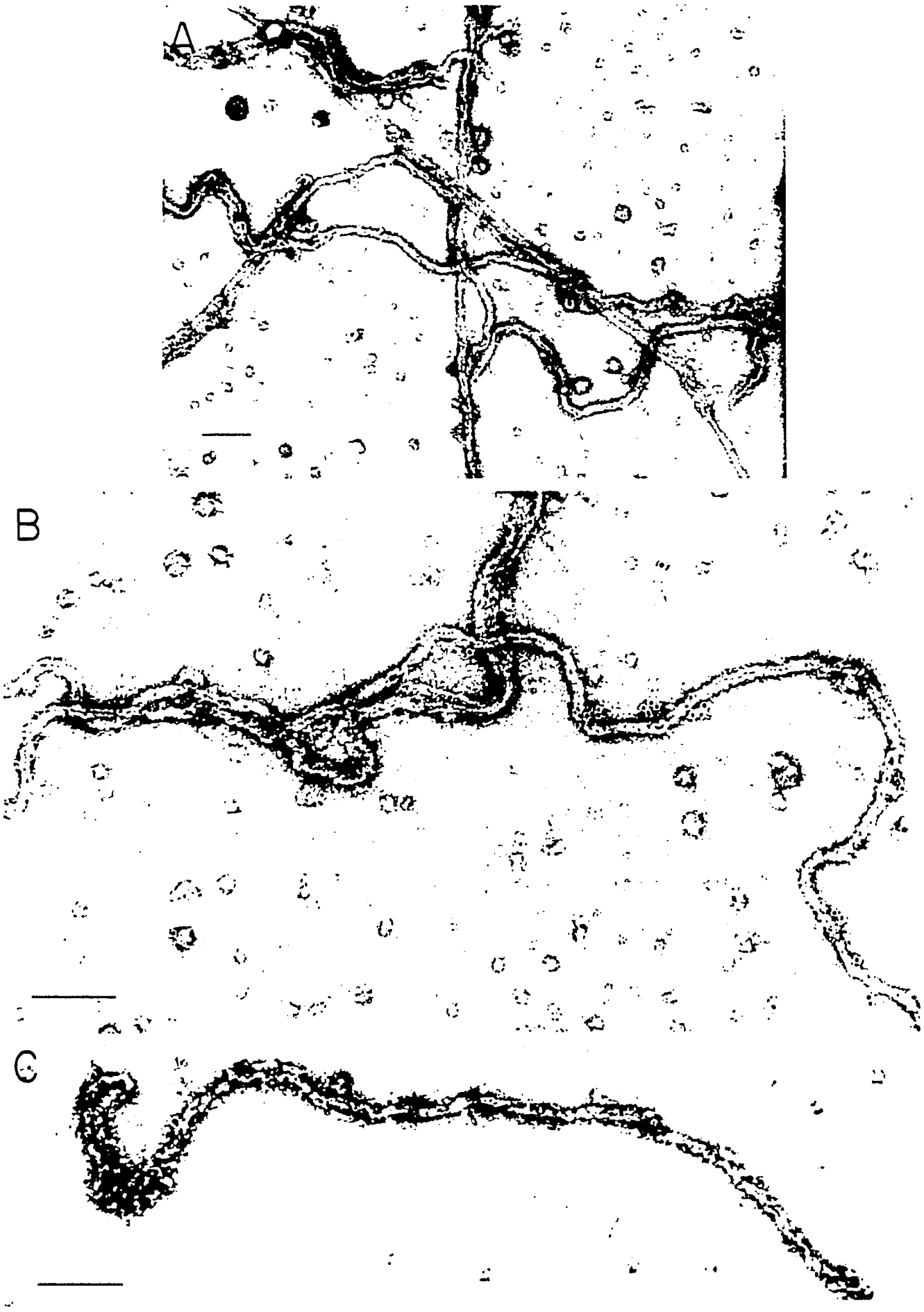


Figure 8

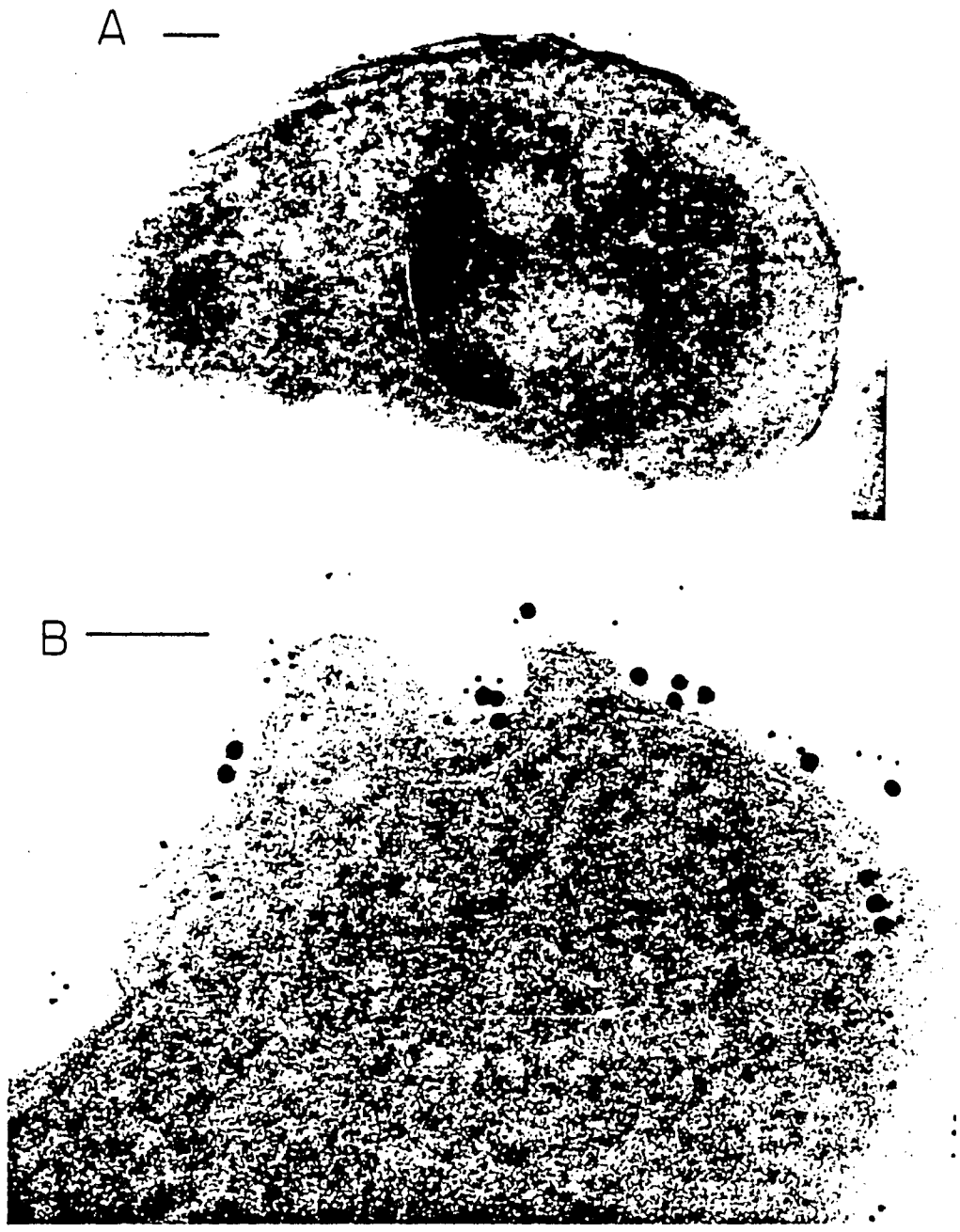


Figure 9: Immunolocalization. *T. brucei* were fixed, cryosectioned and immunolabeled with A. Polyclonal anti p52 antibodies (diluted 1:40 in PBS). Bound antibody was detected using 15nm gold conjugated anti rabbit antibodies. B. Double labeled using anti p52 (diluted 1:30 in PBS) and anti β tubulin (diluted 1:100) antibodies. Bound anti p52 was detected using 15nm gold conjugated anti rabbit antibodies, and bound anti tubulin was detected using 5nm gold conjugated anti mouse antibodies. (A,B, bar, 100nm).

Purification of low molecular weight proteins which bind tubulin

Subpellicular and flagellar microtubules were isolated from *T. brucei* by the use of zwitterion detergent (Zwittergent 3-14) and the subpellicular microtubules were solubilized in the presence of 0.75M NaCl, as described above. When the salt was removed by dialysis, 50% of the solubilized subpellicular microtubules precipitated, and the precipitated material was enriched with a low molecular weight protein which bound tubulin specifically (Fig. 3 lane 3). Since this protein was observed within the precipitated fraction of the dialyzed subpellicular microtubules, it was necessary to isolate the protein without a dialysis step, i.e. while still in solution. The solubilized subpellicular microtubules (in the presence of 0.75m NaCl) were centrifuged through a membrane of a 30kDa cutoff (Centricon 30). The flow through was collected and concentrated on a membrane with a cutoff of 10kDa (Centricon 10). The 10-30kDa proteins collected in this procedure (Fig. 10 lane 1) were dialysed against a low salt buffer, with no precipitate forming.

Affinity purification of microtubule associated protein p15

The tubulin binding protein was purified from the 10-30kDa protein fraction by use of an affinity column. Phosphocellulose purified calf brain tubulin (Fellous *et al.*, 1977) was coupled to Affi gel 10 and the 10-30kDa protein fraction (Fig. 10 lane 1) was incubated with it for 1 hr at 37°C in the presence of 1mM GTP (conditions known to be favorable for microtubule polymerization). The material which did not bind to the column (Fig. 10 lane 2) was washed away, and a 15kDa protein (p15) was eluted from the column upon the addition of 0.75M NaCl (Fig. 10 lane 3).

The purification steps of p52 and p15 are summarized in Appendix 1.

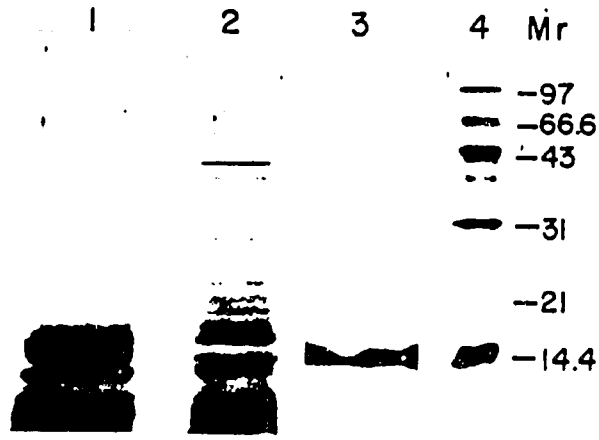


Figure 10. Steps in the purification of p15 - SDS 15% PAGE analysis. Lane 1 shows the 10-30kDa protein fraction which was loaded on the tubulin affinity column. Lane 2 shows the proteins which did not bind to the column. Lane 3 shows the p15 which bound to the column and eluted by 0.75M NaCl. Lane 4 represents molecular weight markers, their molecular mass given in kilodaltons.

Tubulin binding

Tubulin overlay experiments (Kirsch *et al.*, 1989) were carried out in order to confirm that p15 is the tubulin binding protein originally sought for (in the subpellicular microtubule fraction which precipitated upon removal of salt). These precipitated proteins were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and incubated with purified calf brain tubulin in the presence of GTP and in the presence or absence of taxol.

Electroblotted proteins are shown in Fig. 11 lane 1. Tubulin binding occurs in the presence of taxol (Fig. 11 lane 2) but also in its absence (Fig. 11 lane 3). Monoclonal anti tubulin antibodies recognize tubulin only (Fig. 11 lane 4), indicating that the low

molecular weight protein (Fig. 11 lanes 2 and 3) binds tubulin specifically. Polyclonal antibodies raised against p15 recognized a protein of the same molecular weight as the tubulin binding protein (Fig. 11 lane 5), suggesting that indeed p15 is the low molecular weight tubulin binding protein. Of note is the fact that purified p15 bound tubulin specifically on tubulin overlay-dot blot experiments (not shown).

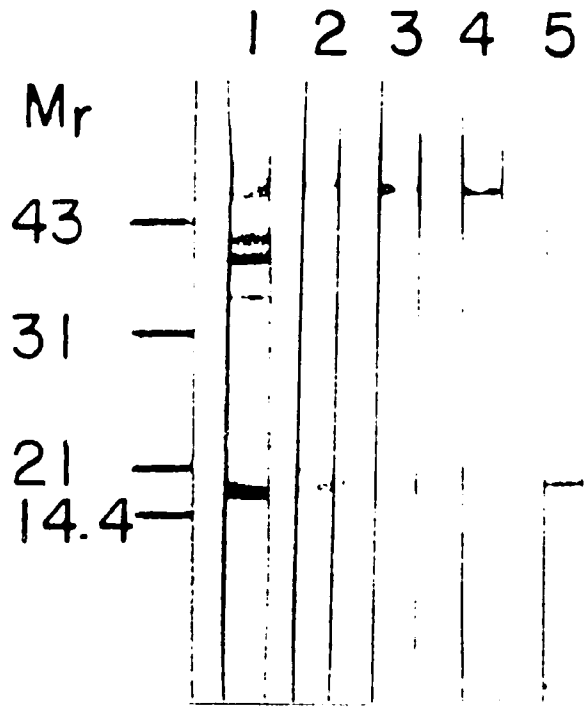


Figure 11. Tubulin binding. Subpellicular microtubules which precipitated during removal of salt were separated on SDS 18% PAGE and electroblotted onto a nitrocellulose membrane. Lane 1 represents the proteins blotted onto the membrane and stained in ponceau. Lane 2 and 3 show the tubulin binding assay. The blotted proteins were incubated in the presence of purified calf brain tubulin, in the presence of GTP and in the presence (lane 2) or absence (lane 3) of taxol. Bound tubulin was detected using anti tubulin antibodies. Lane 4 shows the proteins recognised by anti tubulin antibodies, without prior incubation with tubulin. Lane 5 shows the proteins recognized by anti p15 antibodies. Molecular weight markers are given in kilodalton.

Effect of p15 on tubulin polymerization

Depolymerized calf brain microtubules, containing tubulin and endogenous MAPs (Vallee, 1986), polymerize into single microtubules when incubated in the presence of GTP and taxol (Fig. 12A). When pure calf brain tubulin was incubated with p15 (in the presence of GTP and taxol), the formation of microtubule bundles was observed (Fig. 12B). No crosslinks between the microtubules could be observed even at a higher magnification (Fig. 12C). When p15 was incubated with preformed whole calf brain microtubules, (containing tubulin and endogenous MAPs), some microtubule doublets could be detected (Fig. 12D), suggesting that the binding domain of p15 to tubulin may be distinct from, or additional to, the binding domains already occupied by the endogenous MAPs. The inset shows a high magnification of the doublets formed, where again no crosslinks could be detected. Of note is the fact that without taxol, polymerization did not occur in any of the described polymerization experiments, and without the addition of endogenous MAPs or p15, purified tubulin did not polymerize, even in the presence of taxol.

Figure 12. Microtubules polymerized in the presence of p15 (and taxol and GTP) - negative staining. A: Calf brain microtubules polymerized in the presence of taxol and GTP, without addition of p15. B: Calf brain tubulin polymerized in the presence of p15. C: A high magnification of B. D: Prepolymerized whole calf brain microtubules further incubated in the presence of p15. (A,B,D, bar, 370nm. C,D, bar, 51nm).

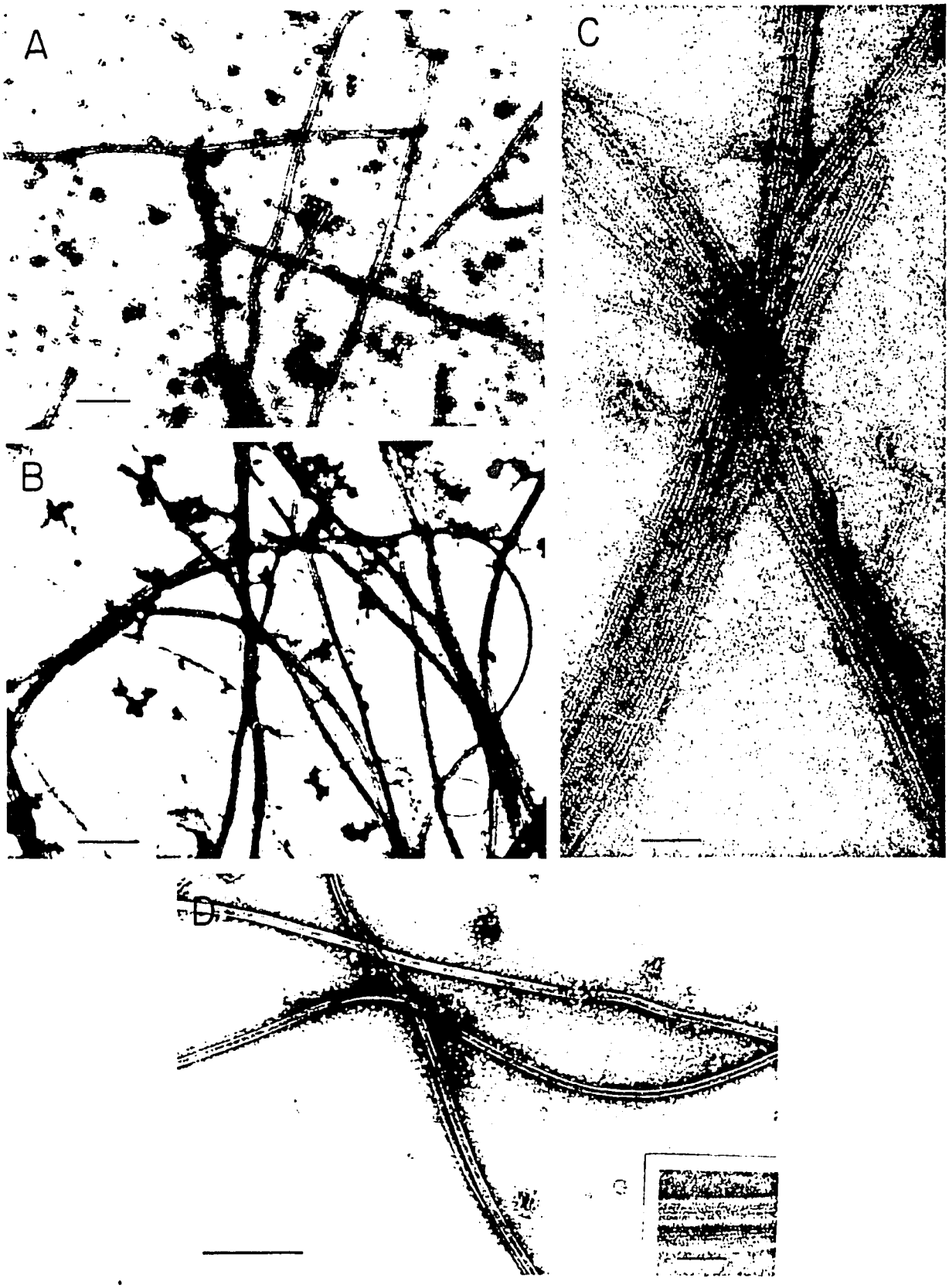


Figure 12

Copolymerization of p15 with tubulin

Calf brain tubulin was polymerized in the presence of the 10-30kDa protein fraction (containing also p15), taxol and GTP, fixed and immunolabeled with antibodies directed against p15 (Fig. 13A) or with monoclonal anti tubulin antibodies (Fig. 13B). Anti p15 antibodies bound to the preformed polymers in a manner similar to that of anti tubulin antibodies, suggesting that indeed p15 has the capacity to copolymerize with tubulin.

Immunolocalization

To determine the localization of p15 in the parasite, we immunolabeled parasites with anti p15 antibodies. Best immunolabeling was achieved using cryosections of 0.1% glutaraldehyde fixed trypanosomes. Since the resolution of the subpellicular microtubules in these sections is poor, we used anti tubulin antibodies as markers for the localization of microtubules. Examination of the cryosections reveals that the major part of the labeling with anti p15 is localized on the periphery of the parasite (Fig. 14A) in a similar labeling pattern to that of anti tubulin antibodies (Fig. 14B), presumably on the subpellicular microtubules. Double labeling using both anti p15 and anti tubulin antibodies (Fig. 14C) reveal the colocalization of anti p15 with anti tubulin.

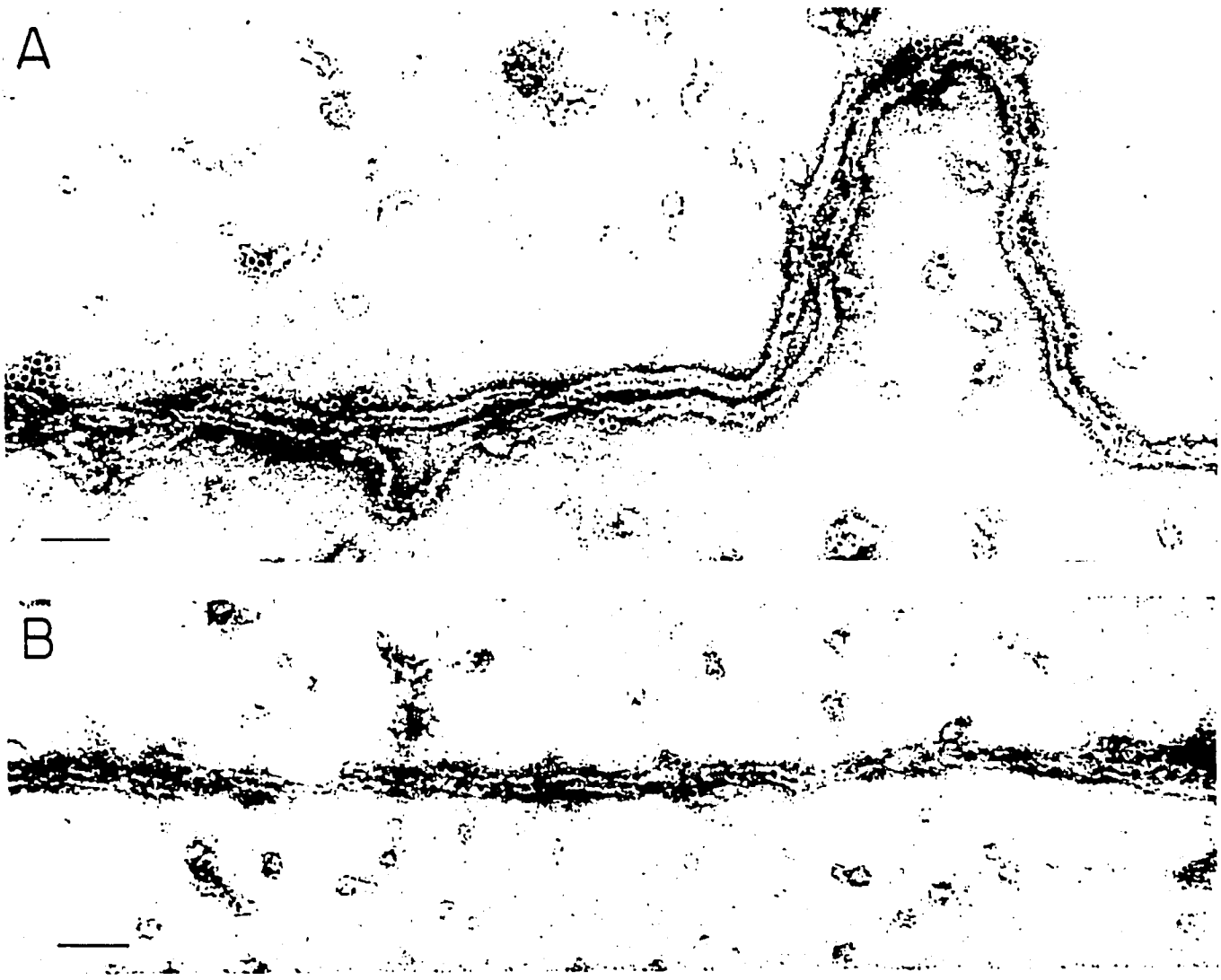


Figure 13. Copolymerization of p15 with tubulin - immunlocalization and negative staining. Calf brain tubulin was polymerized in the presence of the 10-30kDa protein fraction, taxol and GTP, placed on a grid, fixed in 0.1% glutaraldehyde, immunolabeled with anti p15 antibodies (diluted 1:50 in PBS), (A, bar, 100nm), or with anti tubulin antibodies (diluted 1:100 in PBS), (B, bar, 100nm) and negatively stained. Bound anti p15 antibodies were detected using 10nm gold conjugated anti rabbit antibodies and anti tubulin antibodies were detected using 10nm gold conjugated anti mouse antibodies.

Figure 14: Immunolocalization. *T. brucei* were fixed, cryosectioned and immunolabeled with A. Polyclonal anti p15 antibodies (undiluted serum). Bound antibody was detected using 10nm gold conjugated anti rabbit IgG. B. Ponoclonal anti tubulin antibodies (diluted 1:50 in PBS). Bound antibody was detected using 10nm gold conjugated anti rabbit IgG. (A,B, bar, 160nm). C. Double labeled using monoclonal anti tubulin (diluted 1:500 in PBS) and anti p15 antibodies (undiluted serum). Anti tubulin antibodies were detected using 15nm gold conjugated anti mouse IgG, and anti p15 antibodies were detected using 5nm gold conjugated anti rabbit IgG (C, bar, 80nm). flp-flagellar pocket, gly-glycosomes, N-nucleus.

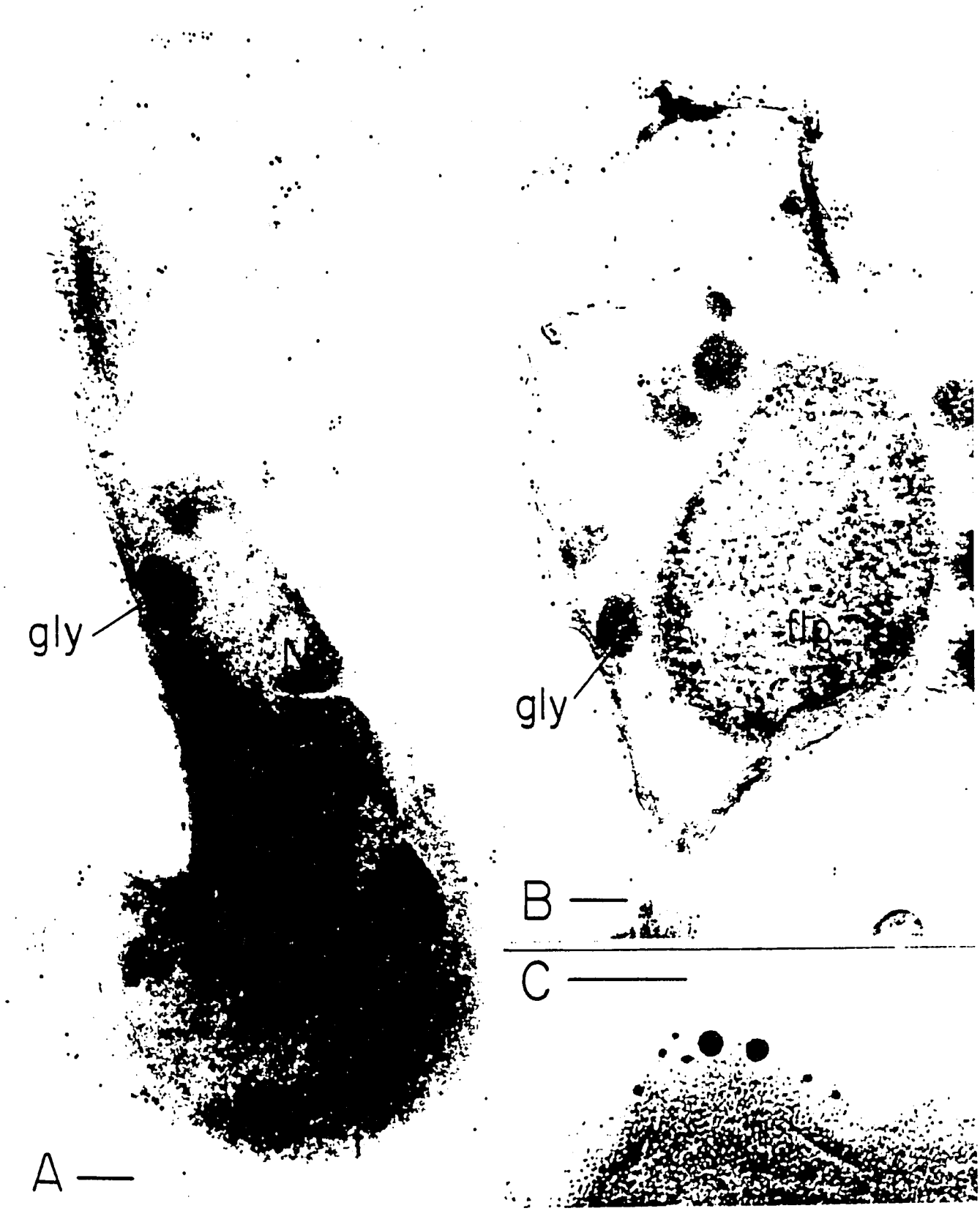


Figure 14

Binding of p15 and p52 to liposomes

Both p15 and p52 are strongly retained on a hydrophobic chromatography matrix (RP 300) and amino acid analysis of these proteins (Appendix 2) reveals a high content of hydrophobic amino acids. These observations suggest the presence of hydrophobic domains in the molecules, available for interaction with phospholipids, and thereby crosslinking between the subpellicular microtubules and the plasma membrane. To explore this hypothesis, we first investigated whether p15 and p52 can actually bind phospholipids. Liposomes were made of soybean asolectin (Seebeck *et al.*, 1988a), incubated with p15 or with p52 for 10 min at 22°C and sedimented by ultracentifugation. The resulting pellet and supernatant proteins were separated by gel electrophoresis and silver stained (Fig. 15).

p15 which was added to the liposomes is shown in Fig. 15A lane 1. Without added liposomes, no precipitate formed (Fig. 15A lane 2), whereas with the addition of liposomes, a precipitate formed which contained p15 (Fig. 15A lane 4). p15 remaining in the supernatant is shown in Fig. 15A lane 3. When 0.25M NaCl (final concentration) was added to the p15-liposome mixture, the binding of the protein to the liposomes was abolished (Fig. 15A lane 6). Fig. 15A lane 5 shows the proteins in the supernatant which were solubilized by the addition of salt. When p52 (Fig. 15B lane 1) was added to the liposomes, the protein was specifically sedimented with the liposomes (Fig. 15B lane 3). The material which did not bind to the liposomes and remained in the supernatant is shown in Fig. 15B lane 2. Addition of salt did not alter the binding of the protein to the liposomes (Fig. 15B lanes 4 and 5 for the non sedimented and sedimented proteins respectively). Without added liposomes no precipitate formed (Fig. 15B lane 6), and the liposomes themselves had no detectable protein content (Fig. 15B lane 7).

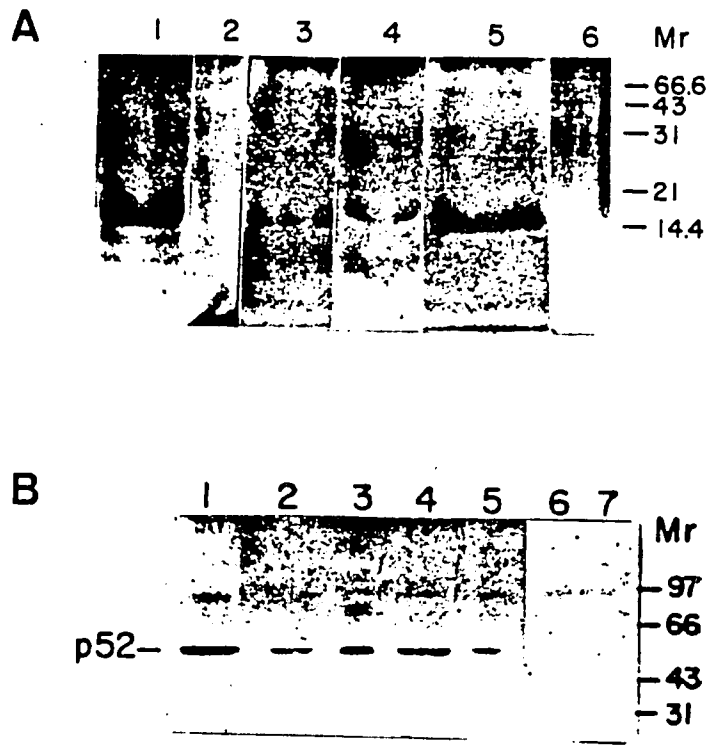


Figure 15: Liposomes were incubated in the presence of p15 (15A) or p52 (15B), sedimented and both supernatant and pellet separated on SDS 15% PAGE (for p15) or SDS 10% PAGE for p52. Fig. 15A: Lane 1 shows p15 without addition of liposomes. Lane 2 shows no precipitate forming without addition of liposomes. Lane 3 shows the proteins remaining in the supernatant after addition of liposomes. Lane 4 shows the proteins sedimented with the liposomes. Lane 5 shows the proteins remaining in the supernatant after addition of liposomes, in the presence of 0.25M NaCl. Lane 6 shows the proteins sedimented with the liposomes in the presence of the salt. Fig. 15B: Lane 1 shows the p52 fraction without the addition of liposomes. Lane 2 shows the proteins remaining in the supernatant after the addition of liposomes. Lane 3 shows the proteins sedimented with the liposomes. Lane 4 shows the proteins remaining in the supernatant after the addition of 0.25M NaCl. Lane 5 shows the proteins which sedimented with the liposomes in the presence of salt. Lane 6 shows the precipitate without the addition of liposomes. Lane 7 shows the liposomes used in these experiments. Molecular weights are given in kilodalton.

Trypanosome proteins mediating binding of microtubules to liposomes

Since p52 and p15 were shown to crosslink microtubules and were also shown to bind liposomes, we checked for their ability to serve as a bridge between polymerized microtubules and liposomes.

Microtubules were polymerized as described above, were then incubated in the presence of soybean asolectin liposomes, and a sample of the mixture was observed under the electron microscope 1 min and 10 min after mixing. Results show that while calf brain microtubules (containing both tubulin and endogenouse MAPs) did not associate with the liposomes (Fig. 16A and B at 1 and 10 min respectively), microtubule polymers made of calf brain tubulin and p15 (Fig. 16C at 1 min) interacted strongly with liposomes, crosslinking them and creating tangles between liposomes and microtubule polymers (Fig. 16D at 10 min incubation). The same was observed for polymers created with p52 (Fig. 16E and 16F for 1 and 10 min incubation respectively).

Figure 16. Crosslinking of liposomes to microtubule polymers-negative staining. A: Whole calf brain microtubules incubated with the liposomes for 1 min (bar, 250nm). B: as in A but microtubules and liposomes were incubated for 10 min (bar, 250nm). C: Microtubule polymers made of calf brain tubulin and p15, mixed with liposomes for 1 min (bar, 450nm). D: as in B but microtubules and liposomes were incubated for 10 min (bar, 450nm). E: Microtubule polymers made of calf brain tubulin and p52, mixed with liposomes for 1 min (bar, 450nm). F: as in D but microtubules and liposomes were incubated for 10 min (bar, 450nm).

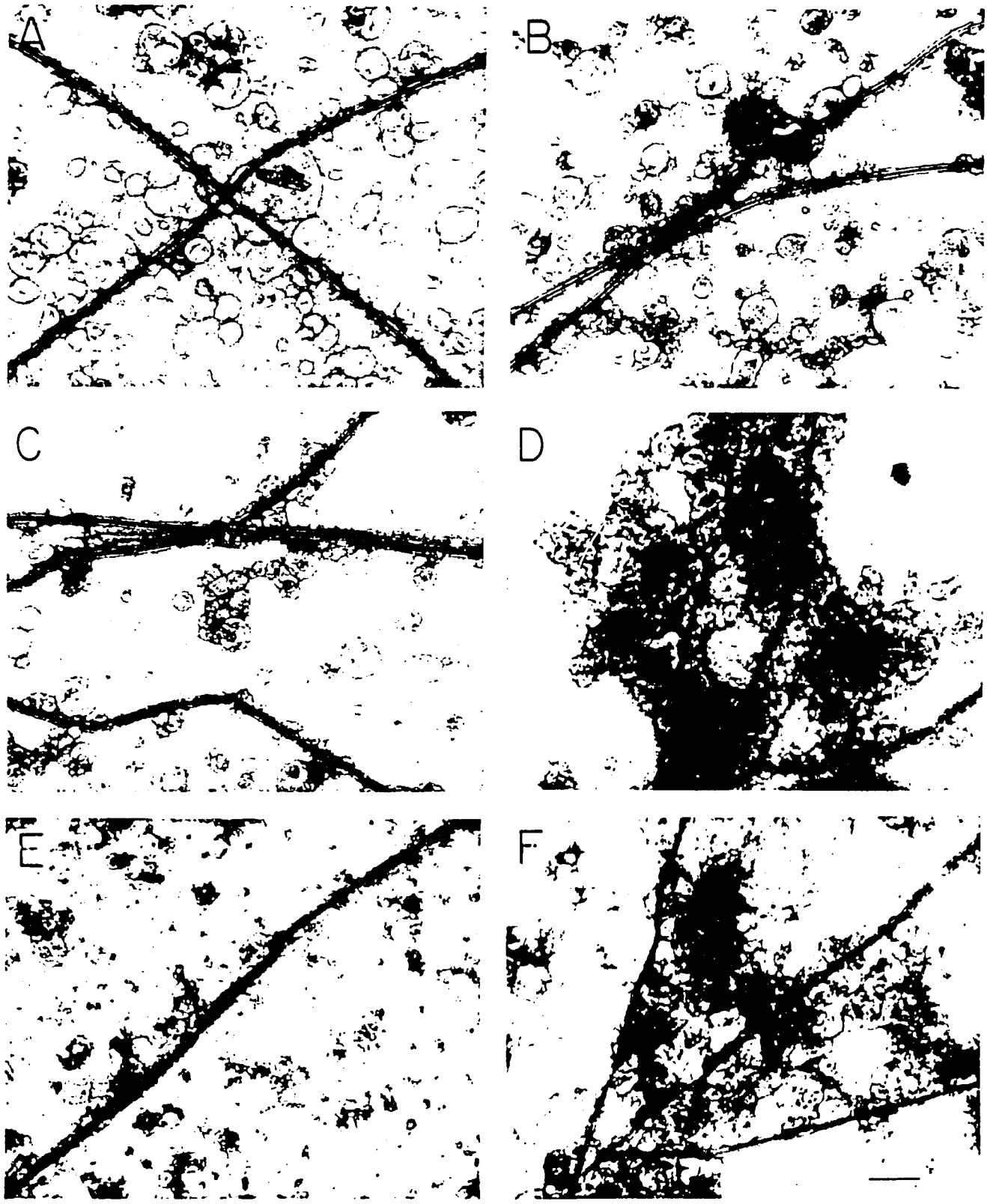


Figure 16

DISCUSSION

The data presented above constitute our findings in an investigation aimed at the understanding of the elements that determine the unique structural organization of the subpellicular network of Trypanosomatidae microtubules. We isolated two subpellicular microtuble associated proteins of 15kDa and 52kDa (p15 and p52 respectively) which have a specific binding capacity for tubulin, can initiate the bundling of calf brain microtubules and can crosslink microtubules to phospholipids.

The subpellicular microtubules of *T. brucei* are cold stable, and the association between the microtubules is likewise cold resistant. Several reports in the literature suggest that the cold stability of Trypanosomatidae microtubules is related to post-translational modifications of tubulin (Gull *et al.*, 1986; Sherwin *et al.*, 1987; Russell & Gull, 1984b; Webster *et al.*, 1987). Detergent treatment of the parasites, solubilized and released from the cytoskeleton cage both the intracellular organelles and the attached plasma membrane. The seemingly rather pure preparation of microtubules that resisted detergent treatment (as seen in the electron microscope) did however contain a rather large number of parasite proteins of both high and low molecular weight. Treatment with 0.75M NaCl released about 50% of these proteins from the insoluble preparation, and upon salt removal by dialysis, a high percentage (50%) precipitated. The fraction that remained in solution was enriched in several proteins and was further purified on a Mono S cation exchange column. Two distinct fractions were eluted, one containing 53kDa protein and the other a 52kDa protein. Of them, the 52kDa protein (p52) had a specific affinity for tubulin and the ability to induce microtubule bundling.

The solubilized subpellicular microtubule proteins which precipitated during removal of salt were found to contain a low molecular weight protein which bound tubulin specifically.

In order to purify this low molecular weight tubulin binding protein, we avoided the precipitation by running the solubilized subpellicular microtubules (still in the presence of salt) through a 30kDa cutoff membrane, and collected a low molecular weight protein fraction which did not precipitate after removal of salt and which still retained the tubulin binding properties. The precipitation which was previously observed was probably due to a specific interaction between tubulin and its associated proteins. Once separated from tubulin by the cutoff membrane, the low molecular weight proteins remained in solution.

A protein of 15kDa (p15) was isolated from the low molecular weight protein fraction by a tubulin affinity column. This protein was found to be a tubulin binding protein in tubulin overlay experiments and was found to bundle microtubules in vitro. Polymerization of tubulin in the presence of p15 resulted in the formation of bundles of microtubules. It is of interest that in contrast to our observation with p52 MAP or with the COPs proteins isolated from *Crithidia fasciculata* (Bramblett *et al.*, 1987; 1989), no crosslinks could be detected between the polymers. The reason for this could be related to the size of p15, isolated in its monomeric form, while the COPs for example, were isolated in their tetrameric form, and could be detected under the electron microscope (Bramblett *et al.*, 1987; 1989).

Antibodies directed against p52 or p15 were found to label microtubule polymers made of subpellicular microtubule proteins, suggesting that indeed these proteins are part of the subpellicular network. p52 and p15 are unique trypanosome proteins; i.e. antibodies directed against them do not crossreact with calf brain microtubules in western blot experiments nor with LA-N1 human neuroblastoma cells in immunofluoresence studies (not shown). Indeed the tight array of the microtubules is a feature unique to the trypanosome subpellicular microtubules, and p52 and p15 may be some of the unique proteins contributing to the special subpellicular microtubule organization.

Our finding of two proteins with a microtubular bundling capacity (p52 and p15) does not exclude the participation of other proteins in the construction of the tight microtubule network (Bramblett *et al.*, 1987; Schneider *et al.*, 1988b; Seebeck *et al.*, 1988b), or in its interaction with the plasma membrane (Seebeck *et al.*, 1988a, Schneider *et al.*, 1988a) and possibly with intracellular proteins.

There have been reports on the ability of mammalian proteins to bundle microtubules in vitro (Kumagai & Sakai, 1983; Aizawa *et al*, 1987). However under our experimental conditions, calf brain microtubules polymerized into single strands and both p52 and p15 trypanosome proteins induced their bundling. The fact that proteins from trypanosomes cause bundling of brain microtubules suggests that the unique structure of the subpellicular microtubules is not a property determined by the tubulin component but is contributed by its associated proteins. The fact that p52 and p15 copurify with subpellicular microtubule proteins, have a specific ability to bind tubulin and are able to induce microtubule bundling, suggests that these are some of the proteins associated with the subpellicular microtubules, giving them their unique form.

The trypanosome subpellicular microtubules are tightly associated with the overlying plasma membrane, suggesting the existence of proteins crosslinking between the two. Since both p52 and p15 showed strong retention on hydrophobic columns and have a high content of hydrophobic amino acids, the possibility that these proteins can interact with the plasma membrane was tested.

The model system used for plasma membrane phospholipids was liposomes made of soybean asolectin. This system was previously used for the study of the interaction of a trypanosome p60 protein with the plasma membrane (Seebeck *et al.*, 1988a). We show that p52 and p15 can interact with liposomes and crosslink between preformed microtubules

and liposomes. The association of p15 with the liposomes is abolished in the presence of 0.25M NaCl, thus indicating electrostatic interactions between the two. This interaction does not exclude additional hydrophobic interactions between p15 and the liposomes. Electrostatic interactions are important for the initial contact of peripheral membrane proteins with the lipid bilayer, so that their neutralization could reduce subsequent hydrophobic interactions (Cullis *et al.*, 1985). The association of p52 with the liposomes was not abolished by salt, suggesting that the interaction of p52 with the liposomes is more of a hydrophobic nature.

Our results indicate that p52 and p15 may have a double role of both stabilizing the microtubules and crosslinking the microtubules to the plasma membrane, giving the subpellicular microtubules their unique and stable architecture.

II. The association of glycosomal enzymes and microtubules: A physiological phenomenon or an experimental artifact?

INTRODUCTION

The glycosomes are peroxisome-like bodies which are unique to *Kinetoplastidae.* The glycosomes contain several enzymes involved in glycolysis and glycerol metabolism, as well as enzymes involved in diverse metabolic pathways such as carbon dioxide fixation, pyridine biosynthesis, ether-lipid biosynthesis and purine salvage (Opperdoes, 1987). The blood-stream form of trypanosomes possesses only a rudimentary mitochondrion and derive its energy from substrate-level phosphorylation during glycolysis (Fairlamb & Opperdoes, 1986). Compartmentalization of the glycolytic enzymes within the glycosomes facilitates the latter process (Opperdoes *et al.*, 1984).

The microtubules of the trypanosome are found in various domains of the parasite. In the mitotic spindle, in the flagellar axonemas, in the cytoplasm and in the subpellicular area. The subpellicular microtubules create a cage-like structure of parallel microtubules, which are interconnected laterally and whose course is in close apposition to the overlying plasma membrane (Seebeck *et al.*, 1988b).

A direct interaction between subpellicular microtubules and glycosomal enzymes *in vivo* seems unlikely, since the bulk of glycosomal enzymes is known to be restricted to the glycosome interior (Hart *et al.*, 1987) and since the cytosolic stage of glycosomal enzymes is short (less than 5 min (Clayton, 1987)). However, evidence for interactions between cytoskeletal proteins and glyclytic enzymes (Clarke & Masters, 1975) is accumulating. For example, GAPDH was found to bundle preformed microtubules in the presence of DMSO

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(Kumagai & Sakai, 1983), to interact with F-Actin (U. Certa, personal comunications) and to be localized histochemically in the isotropic zones of cross striated muscle (Arnold & Pette, 1968). Aldolase and phosphofructokinase (PFK) have been shown to associate with myofibrils (Liou & Anderson, 1980; Choate *et al.*, 1985). A 41kDa COP protein was isolated from *Crithidia fasciculata* along with microtubules and was found to be the glycosomal enzyme GAPDH (Bramblett *et al.*, 1989), and a trypanosome p60 glycosomal enzyme was found to interact both with microtubules and with lipids (Seebeck *et al.*, 1988a; Kueng *et al.*, 1989).

Here we show that glycosomal enzymes such as aldolase and GAPDH copurify with trypanosomal subpellicular microtubules, cosediment with polymerizing brain tubulin, and that aldolase is able to crosslink microtubules *in vitro*. We discuss whether the association between glycosomal enzymes and microtubules may be of physiological relevance, and consider the significance of these observations for the set of criteria commonly used to identify microtubule-associated proteins (MAPs). To clarify the latter issue, we compare the association of glycosomal enzymes with microtubules to that of p52.

RESULTS

Purification of proteins which associate with the subpellicular microtubules

The purification of *T. brucei* subpellicular microtubules was described above. Briefly, *T. brucei* (Fig. 17 lane 1) were subjected to zwitterion detergent (Zwittergent 3-14) to remove plasma membrane and soluble macromolecules, and the insoluble material was collected by centrifugation (Fig. 17 lane 2). This insoluble fraction which electron microscopic inspection showed to consist of flagellar and subpellicular microtubules (Fig. 1B), was partially (50%) solubilized by 0.75M NaCl. The soluble fraction (Fig. 17 lane 3) was enriched in subpellicular microtubule proteins since the insoluble fraction (Fig. 17 lane 4) consisted of flagellar microtubules (as observed under the electron microscope (Fig. 1C). Next, salt was removed during which 50% of the proteins reprecipitated. The proteins remaining in solution after removal of salt (Fig. 17 lane 5) were fractionated on a phosphocellulose cation exchange column. This column had been chosen because of its conventional use for separating between tubulin and its associated proteins (Fellous, *et al.*, 1977). Indeed tubulin ran through the column (Fig. 2 lane 6), and its associated proteins were eluted from the column as a one peak fraction by a 0-1M NaCl gradient. This fraction (eluted at around 0.5M NaCl) contained 3 proteins of 52, 41 and 36kDa (Fig. 17 lane 6, and purification scheme, Appendix 1, VI).

Identification of the proteins eluted from the phosphocellulose column

The phosphocellulose purified proteins were separated by gel elecrophoresis and each was electroeluted individually from the gel. The 36kDa protein was found by amino acid sequence analysis of the ten amino terminal amino acids to be the glycosomal enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH), (EC 1.2.1.12). The two higher molecular weight proteins were amino terminally blocked and neither CNBr cleavage,

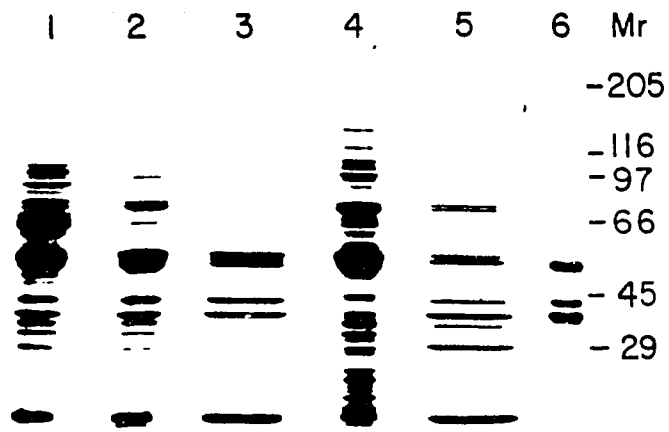


Figure 17. Steps in the isolation of *T. brucei* microtubule proteins-SDS 5-15% PAGE analysis. Lane 1 shows the spectrum of proteins in a total trypanosome homogenate. Lane 2 shows the proteins which were not solubilized by detergent. Lane 3 shows the proteins which were solubilized by NaCl. Lane 4 shows the proteins which were not solubilized by salt. Lane 5 shows the proteins which were solubilized by NaCl and which remained soluble after removal of salt. Lane 6 shows the proteins that were retained on the phosphocellulose column and were eluted from it by NaCl. Molecular weight markers are given, their molecular mass in kilodalton.

Table 1 ALDOLASE DISTRIBUTION DURING PURIFICATION OF TRYPANOSOME MAPs

Fraction tested	Aldolase activity units/mg protein
Total cell homogenate (Fig. 1 lane 1)	3.0
Detergent insoluble proteins (Fig. 1 lane 2)	1.8
Salt insoluble proteins (Fig. 1 lane 4)	0.2
Salt solubilized proteins which remained soluble after salt removal (Fig. 1 lane 5)	12.8
Proteins precipitated during salt removal	0.6
Phosphocellulose purified proteins (Fig. 1 lane 6)	32.0

trypsinization nor V8 digestion proved satisfactory for generation of peptide fragments suitable for amino terminal sequencing. However, since they had a molecular mobility similar to that of aldolase and phosphoglycerate kinase (PGK) (Opperdoes, 1987), we tested the possibility that either one or both were also glycosomal enzymes. We therefore checked the phosphocellulose eluted proteins for their enzymatic activity. PGK activity was not detected in this fraction but the fraction was greatly enriched with aldolase activity (see Table 1). Additional evidence for the presence of aldolase was provided by ELISA experiments where antibodies directed against trypanosome aldolase (a generous gift of C. Clayton) cross-reacted with the phosphocellulose purified fraction (not shown).

In order to exclude the possibility that p52 is PGK, a comparison of the proteolytic fragment of PGK (Fig. 18 lane 1 and 2) was compared to that of p52 (Fig. 18 lane 3 and 4) by the use of V8 digestion. We used the commercially available yeast PGK since it has a high (50%) sequence homology with trypanosome PGK (Weirenga *et al.*, 1987). No similarities were detected between the peptides formed. In order to further exclude the possibility that p52 is a glycosomal emzyme, it was run with the kind assistance of M. Parsons on two-dimentional gel electrophoresis. The protein did not show identity of mobility with any known glycosomal enzyme (data not shown).

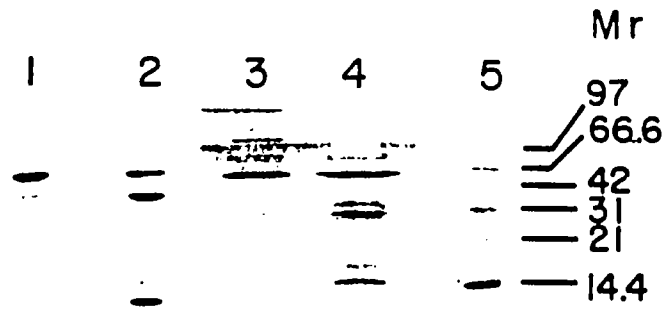


Figure 18. Proteolytic digestion of PGK (lanes 1,2) and p52 (lanes 3,4): SDS 15% PAGE analysis. Lane 1 shows PGK without added V8. Lane 2 shows PGK digested with the enzyme. Lane 3 shows p52 without added enzyme. Lane 4 shows p52 digested with the enzyme. Lanes 1,2 and 5 were stained with coomassie while lanes 3 and 4 were silver stained. Lane 5 represents the molecular weight markers, their molecular mass in kilodalton.

Taxol precipitation

Taxol is an antimitotic drug known to promote and stabilize microtubule assembly (Vallee, 1982), and is often used for the determination and purification of molecules associated with tubulin (Vallee, 1986). The solubilized (and dialyzed) subpellicular microtubules (Fig. 19 lane 1) were incubated in the presence of phosphocellulose purified calf brain tubulin (Fig. 19 lane 2), taxol and GTP. The precipitate that formed was analyzed by gel electophoresis and showed that tubulin precipitated along with proteins of 52kDa, 41kDa and 36kDa (Fig. 19 lane 3). Westen blot analysis of the taxol precipitated proteins (Fig. 20 lane 1) showed that anti aldolase antibodies recognized the 41kDa protein (Fig. 20 lane 4) and that anti GAPDH antibodies recognized the 36kDa protein (Fig. 20 lane 5). The position of tubulin and p52 are shown in Fig. 20 lanes 2 and 3 respectively, using anti tubulin and anti p52 antibodies respectively.

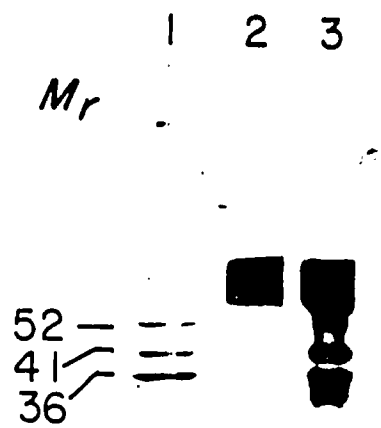


Figure 19. Taxol induced precipitation-SDS 10% PAGE analysis. Lane 1 shows the solubilized subpellicular microtubules to be precipitated. Lane 2 shows the purified calf brain tubulin. Lane 3 shows the proteins precipitated in the presence of calf brain tubulin, taxol and GTP. Major polypeptides are identified by their molecular mass in kilodalton.

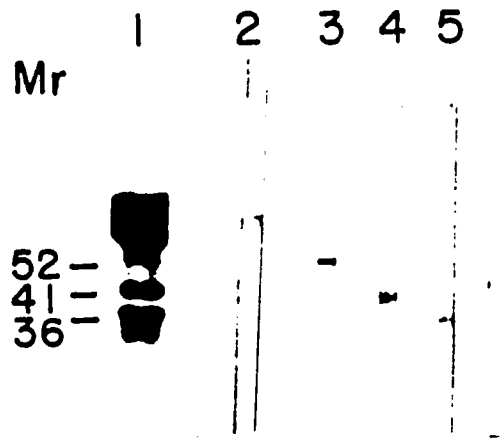


Figure 20. Western blot analysis of taxol precipitated proteins. Taxol precipitated subpellicular microtubules (in the presence of tubulin taxol and GTP) were separated on SDS 10% PAGE, electroblotted onto a nitrocellulose membrane and incubated with the antibodies. Lane 1 shows the proteins which were electroblotted and stained in ponceau. Lane 2 shows the binding of anti tubulin antibodies. Lane 3 shows the binding of anti p52 antibodies. Lane 4 shows the binding of anti aldolase antibodies and lane 5 shows the binding of anti GAPDH antibodies. Major polypeptides are identified by their molecular mass in kilodalton.

Microtubule polymerization

Calf brain microtubules polymerized *in vitro* in the presence of taxol and GTP, form long strands of single microtubules (Fig. 7A). Purified calf brain tubulin incubated in the presence of the phosphocellulose eluted proteins containing 52kDa protein (p52), aldolase and rabbit GAPDH (in the presence of taxol and GTP), polymerized into a structure which is similar to the original form of the subpellicular microtubules, i.e. of microtubule bundles crosslinked to one another (Fig. 21A). To assess the ability of each of these proteins to bundle and crosslink microtubules, we repeated the polymerization experiments with each protein separately. p52 was purified on a Mono S cation exchange column, and aldolase and GAPDH were used from commercially available rabbit aldolase and GAPDH (which have a high sequence homology with trypanosome enzymes (Weirenga *et al.*, 1987)).

Results show that while GAPDH had no polymerization or bundling capacity (not shown), aldolase crosslinked microtubules (Fig. 21B) in a manner similar to the phosphocel-

lulose eluted fraction (containing all 3 proteins), and to the polymerization pattern observed with p52 (Fig. 7C). Of note is the fact that commercially available yeast PGK did not polymerize microtubules, in support of our conclusion that p52 is not the glycosomal enzyme PGK.

Without the addition of taxol no polymerization occurred in any of the described polymerization experiments, and without the addition of the phosphocellulose fraction, aldolase or p52, tubulin had no polymerization capacity (not shown).

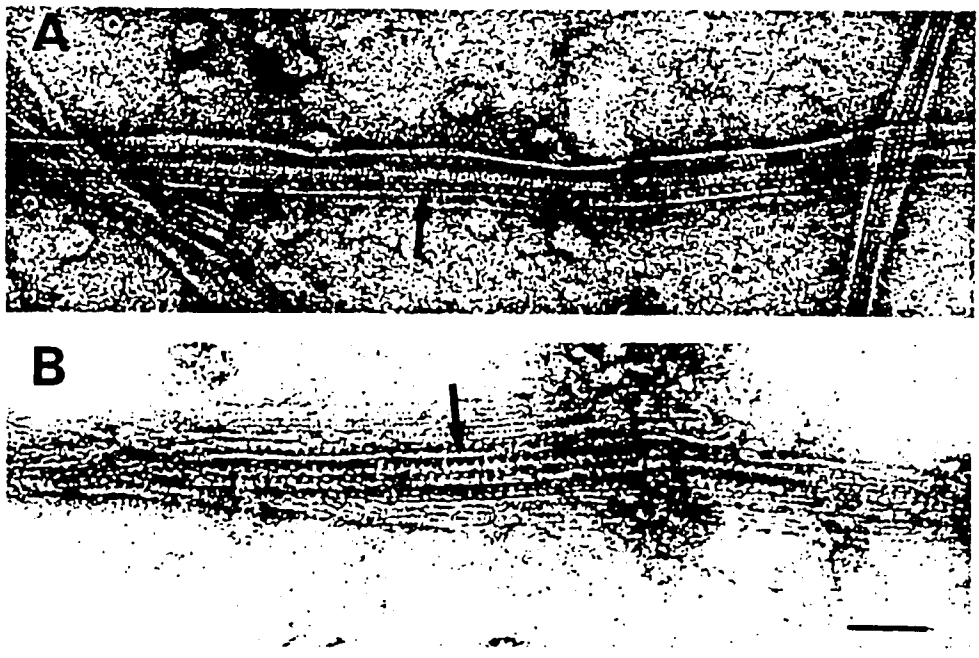


Figure 21. Polymerization studies-negative staining. Calf brain tubulin was polymerized in the presence of phosphocellulose purified trypanosome proteins (A) or in the presence of commercially available rabbit aldolase (B). (A,B, bar, 56nm). Arrows indicate the location of crosslinks.

Immunolocalization

To investigate the possibility of a physiological association between glycosomal enzymes and subpellicular microtubules, we performed immunolabeling studies with antibodies directed against trypanosome aldolase and GAPDH, and compared them with antibodies directed against p52 as well as with antibodies directed against p15.

Best immunolabeling was achieved using cryosections of 0.1% glutaraldehyde fixed trypanosomes. Since the resolution of the subpellicular microtubules in these sections is poor, we used anti tubulin antibodies as markers for the localization of microtubules.

Two distinct patterns of immunolabeling were observed. Antibodies directed against aldolase (Fig. 22A) and against GAPDH (Fig. 22B) labeled glycosomes exclusively, whereas antibodies directed against p52 (Fig. 22C) and against p15 (Fig. 22D) did not label the glycosome but their labeling resembled that of anti tubulin antibodies (Fig. 22E), i.e. of the subpellicular microtubules.

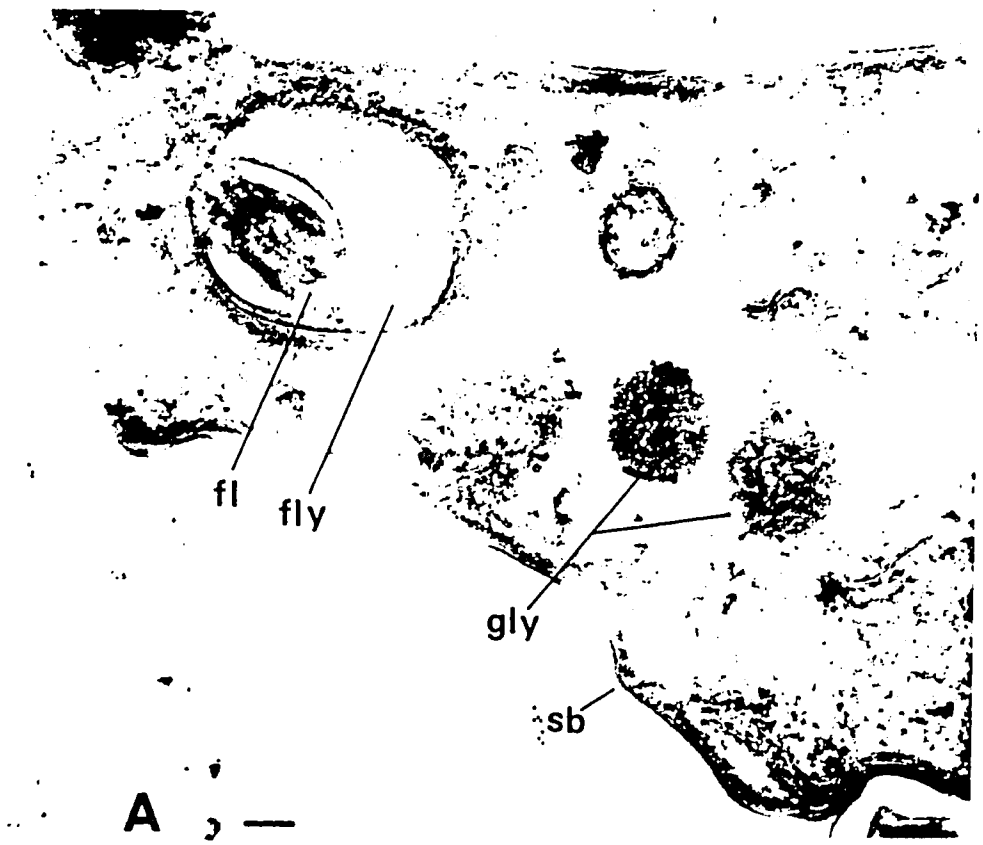


Figure 22. Immunolocalization. *T. brucei* were fixed, cryosectioned and immunolabeled by A. Polyclonal anti trypanosome aldolase antibodies (bar, 107nm). B. Monoclonal anti trypanosome GAPDH antibodies. C. Polyclonal anti p52 antibodies. D. Polyclonal anti p15 antibodies. E. Polyclonal anti tubulin antibodies (B-E, bar, 107nm). Bound antibodies were detected using 10nm gold conjugated anti mouse (B,C) or anti rabbit (A,D,E) IgG. flp-flagellar pocket, fl-flagella, gly-glycosome, sb-subpellicular microtubules.

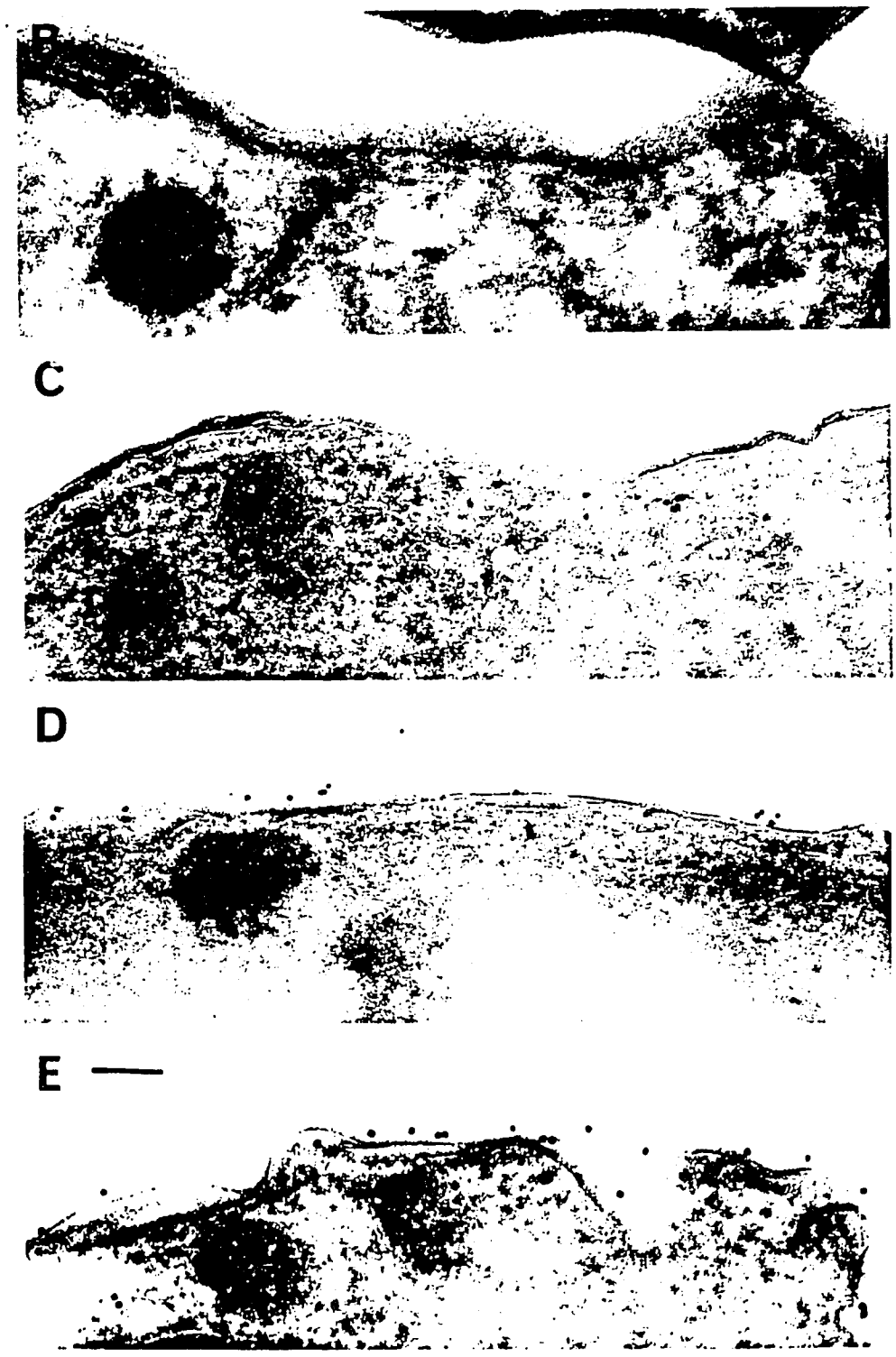


Figure 22

DISCUSSION

The results presented in this chapter show that the glycosomal enzymes aldolase and GAPDH co-purify with trypanosome subpellicular microtubules, and are specifically co-sedimented with calf brain tubulin after a taxol induced tubulin polymerization. We also show that rabbit aldolase (which has an about 50% sequence homology with trypanosome aldolase (Weirenga *et al.*, 1987)) can induce microtubule crosslinking *in vitro*. These observations could suggest a possibility of a physiological association between glycosomal enzymes and microtubules, as has been shown to exist for example between the glycosomal enzyme phosphofructokinase (PFK) and myofibrils (Choate *et al.*, 1985).

The microtubules could serve as regulatory proteins for the transport of glycolytic enzymes into the glycosomes since these enzymes are synthesized in the cytosol and are posttranslationally transferred to the glycosomes (Clayton, 1987; Hart *et al.*, 1987). However, to our knowledge, no evidence for a microtubule-bound form of aldolase and GAPDH has been reported. Both enzymes are known to exist both as cytosolic and as glycosomal entities. However, the cytosolic state is transient (turnover less than 5 min (Clayton, 1987)), and this turnover rate would seem to be inconsistent with the significant population of microtubule-bound enzymes during purification of the subpellicular microtubules. In addition, the data we obtained from immunolabeling of aldolase and GAPDH in the trypanosome, clearly indicates, that at the detection level, both enzymes are by and large restricted to the glycosomes. A physiological association between the two glycosomal enzymes and the subpellicular microtubules thus appear unlikely.

Alternatively, the co-purification of aldolase and GAPDH with the subpellicular microtubules, may be a results of a similar behavior of these proteins during the purification procedure. Upon cell lysis with detergents, the glycosomal enzymes form multi enzyme

insoluble complexes which are solubilized by high salt (Misset *et al.*, 1986). Thus when plasma membranes and glycosome membranes are solubilized by the detergent, the glycosomal enzymes may cluster and cosediment with the subpellicular microtubules. Moreover, in the next step of the purification, the salt solubilization, proteins of both elements go into solution. This behavior would result in the copurification of the enzymes with the subpellicular microtubule proteins, even though no tubulin-enzyme association takes place.

The glycosomal enzymes have a net positive charge (Opperdoes, 1988), and are therefore expected to be retained on phosphocellulose columns and to be eluted from the column by salt, just like microtubule associated proteins (MAPs) (Fellous *et al.*, 1977). Furtheremore, since phosphocellulose is a β 1,4-glucose polymer containing phosphate groups at position 2 and 6, the column may also have a property of serving as an affinity column for aldolase and GAPDH (Barnard & Pederson, 1988), since the substrate for aldolase is fructose 2,6 diphosphate, and the substrate for GAPDH is glyceraldehyde phosphate. Phosphocellulose might therefore retain these enzymes by virtue of its specific affinities and net charge properties.

Finally, the observed co-purification and co-sedimentation of aldolase and GAPDH with tubulin, and the ability of aldolase to promote tubulin polymerization and to crosslink microtubules, could reflect a non-specific enzyme-tubulin binding. All glycosomal enzymes are known to be positively charged (Opperdoes, 1988), and aldolase and GAPDH are known to carry at least two clusters of net positive charge known as hot spots (Wierenga *et al.*, 1987). Conceivably, these hot spots might serve as non specific binding domains for negatively charged tubulin molecules.

To qualify as a true MAP, proteins should copurify with tubulin, cosediment with it upon polymerization into microtubules and promote tubulin polymerization (Olmsted, 1986).

Since GAPDH and aldolase seem to fullfil these criteria, and the immunolocalization of these proteins does not support an *in vivo* association with microtubules, the definition of a MAP would seem to require additional parameters. If we compare the behavior of the three proteins described in the present report, we find that even though the three copurify with the trypanosome microtubules and cosediment with polymerizing brain tubulin, they have a distinctly different location within the cell, and only p52 seems to localize on the subpellicular microrubules and to bind tubulin on tubulin overlay experiments. Similarly p15 localizes on the subpellicular microtubules and binds tubulin on tubulin overlay experiments. We thus suggest that while p52 and p15 may be true MAPs, or that their association with tubulin may have a physiological significance, the association of GAPDH and aldolase with tubulin may be a result of the experimental system, and we would therefore like to point out the caution one should take before suggesting true associations between tubulin and other molecules.

III. Trypanosome microtubule associated proteins - A possible vaccine against trypanosomiasis

INTRODUCTION

Trypanosomes enter the bloodstream where they exhibit a remarkable power of adaptation, allowing them to escape the host immune response. The parasites have evolved a mechanism of antigenic variation, where the trypanosome is able to undergo an apparently inexhaustible series of variations of its surface antigens (VSG) and so evades the host's immune response (Vickerman & Luckins, 1969; Borst & Cross, 1982; Van der Ploeg, 1987). Accordingly the number of parasites in the blood fluctuates due to the destruction of the trypanosomes by the host, only for the emergence of new parasites of different antigenic constitution. As a result, the parasite is able to survive within the host and produce an ongoing disease as well as an immunological situation that has thus far thwarted the creation of an effective vaccine. The large repertoire of variable surface antigens which can be expressed by a single parasite made vaccination appear as a goal beyond reach. Some vaccination studies have been done using flagellar pocket-nonvariant trypanosome proteins. However, only partial protection was achieved (Olemnick *et al.*, 1988).

Since the development of a vaccine appeared beyond reach, emphasis has been placed on the development of improved drug therapy directed against vital metabolic processes that are unique to the trypanosome. However, efforts to produce trypanocidal drugs have also been only partially successful. Drugs existing today are sometimes not effective or are too toxic and cause side effects (Fairlamb & Bowman, 1980; Opperdoes, 1983; c.f. Molyneux & Ashford, 1983).

Trypanosomiasis control programs aimed at eradication of the most common vector, the tsetse fly, though adequate if well managed, have not been fully effective (Odino, 1988; Owaga *et al.*, 1988).

It is therefore essential to find a new specific target site for therapeutic attack.

All parasitic trypanosomes contain a prominent subsurface array of microtubules unique in localization and apparent stability. This suggests that the functional integrity of trypanosome microtubules may be important in the control and maintenance of membrane-located physiological processes that are essential for the survival of the parasite in the host (Baum *et al.*, 1981). The unique trypanosomal MAPs could serve as therapeutic target sites, taking advantage of the importance of the microtubule integrity to the parasites and the uniqueness of these MAPs to trypanosomes.

Unique trypanosomal MAPs could not only serve as chemotherapeutic target sites, but may also serve as unique antigens for immunological supression of the disease. In our study we explored the possibility of using intracellular antigens as candidates for immunological supression of the disease.

To our knowledge, the use of intracellular antigens as vaccines has never been considered. However, immunofluorescence studies have been described (Shlomai & Zadok, 1984), where the intact trypanosomatid *Crithidia fasciculata* was incubated in the presence of fluorescent antibodies directed against kinetoplast DNA, and fluorescence was detected within the parasite. Furthermore, uptake of macromolecules has been recorded in the trypanosome (Coppens *et al.*, 1985 & 1987, Opperdoes *et al.*, 1987). Molecules such as ferritin (Brown *et al.*, 1965; Langreth & Balber, 1975; Steinert & Novikoff, 1960; Webster & Grabb, 1988), Vinylpyrrolidone (Fairlamb & Bowman, 1980), Triton WR-1339 (Opperdoes & Van Roy, 1982) and total serum albumin were taken up by a mechanism of fluid endo-

cytosis, while LDL and transferrin were taken up by a receptor mediated process (Coppens *et al.*, 1987).

In preliminary experiments, complete protection was obtained against a *T. brucei* infection when animals were vaccinated with a unique trypanosomal p52 MAP together with the glycosomal enzymes aldolase and GAPDH (which copurified along with the p52). Sera of protected animals caused the aggregation of trypanosomes *in vitro*, and immunoelectron microscopy of these aggregated trypanosomes revealed the presence of antibodies within the parasites.

RESULTS

The antigen

The proteins used for vaccination were a mixture of the trypanosome p52 MAP and the glycosomal enzymes aldolase and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) which copurified along with p52 on phosphocellulose columns. The purification steps are described in Fig. 2, and the proteins used for vaccination are shown in Fig. 2 lane 7 (Purification scheme, appendix 1, VI).

Vaccination studies

The proteins eluted from the phosphocellulose column (Fig. 2 lane 7) were injected into rats and mice which were then challenged with *T. brucei*. The schedule of immunization and the protection afforded are summarized in Table 2. Table 2 shows that a single injection of antigen did not induce protection. Two injections did, however, partially protect mice and rats against *T. brucei*. The protection reached 100% when the vaccination schedule consisted of three injections of antigen. The surviving animals were followed for 60 days without development of parasitemia. Control mice and rats died within 5-12 days.

The antibody level in the blood of vaccinated animals against the injected antigen was monitored weekly by ELISA. Fig. 23 shows that the antibody level reached a plateau value after the third antigen injection, and persisted at this level for at least 60 days.

It is known that *T. brucei* is sometimes harboured in the brain (Waitumbi *et al.*, 1988), and therefore when brains of infected mice are injected into irradiated mice, they develop parasitemia. In order to eliminate the possibility of a hidden parasitemia, brains of vaccinated and protected mice were inoculated into a group of irradiated mice. Parasitemia did not appear during 30 days of observation, suggesting that the brains of vaccinated animals were also protected.

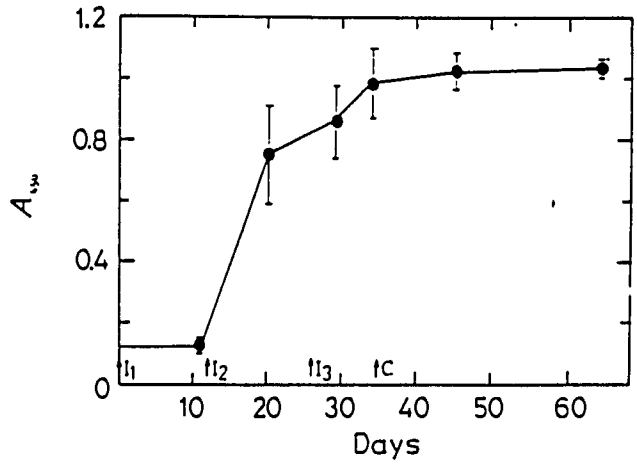


Figure 23. Development of antibodies to the injected antigen. Four rats were injected three times with the antigen on the specified days (I_1, I_2 and I_3) and challenged with *T. brucei*. (C). Blood was withdrawn at specified times, and antibody titer in a 1:100 dilution of serum was determined by ELISA against the injected antigen, using peroxidase linked anti rat antibodies. Results are means of the absorbance at 450nm ± standard error of the mean.

Phosphocellulose purified proteins of other trypanosomes

A phosphocellulose column retained and salt eluted protein fraction was also obtained from *T. rhodesiense* (KETRI 1886, an acute human strain) and *T. evansi* (EATRO 1342, an acute camel strain). This fraction also consisted of three proteins of 52kDa, 41kDa and 36kDa, as those purified from *T. brucei*. The antibodies developed in animals vaccinated with *T. brucei* proteins cross reacted with proteins of these other trypanosome species in ELISA experiments (not shown).

TABLE 2: IMMUNIZATION AGAINST T.BRUCEI INFECTION BY THE PROTECTION INDUCING ANTIGEN

			MICE			RATS		
Exp.	Antigen injection on day	Day of * challenge with T.brucei	Parasitemia; day after challenge (no.of mice)	Day of ** death (no.of mice)	Survival live/total (% protection)	Parasitemia; day after challenge (no.of rats)	Day of death (no.of rats)	Survival ** Live/total (% protection)
Controls	---	see *	5 (8)	7-8 (8)	0/8 (0)	5 (8)	8-12 (8)	0/8 (0)
1	0	8	5 (4)	7-8 (4)	0/4 (0)	5 (4)	8-12 (4)	0/4 (0)
2	0 and 7	11	5,13 (1)(1)	7,15 (1)(1)	4/6 (66)	13 (1)	20 (1)	4/5 (80)
3	0,12 and 24	9	-	-	4/4 (100)	-	-	4/4 (100)

* Counted from the day of the last injection of antigen. Controls challenged on the same day.
** At least for 60 days after challenge.

Further vaccination studies

Vaccination studies were repeated several times but no protection was achieved. Various phosphocellulose fractions were injected, rabbit aldolase or rabbit GAPDH were injected separately, different schedules of immunizations were tried as well as different locations of immunizations (with the rationale that in the case of *Leishmania* antigens, subcutaneous injections exacerbate the disease while intravascular injections suppress the disease (Liew *et al.*, 1985)), but without success. We also compared the sera of vaccinated and protected (Fig. 24A lanes 1-4) and non protected animals (Fig. 24A lanes 5-8) on western blots, but no obvious difference was found. Preimmune sera of these animals showed no labeling at all (not shown).

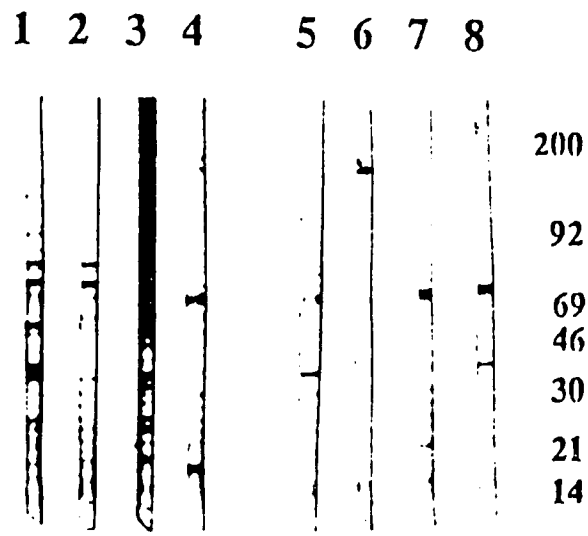


Figure 24. *T. brucei* antigens recognized by sera of vaccinated and protected (lanes 1-4) or non protected (lanes 5-8) mice. Total trypanosome proteins separated on SDS 5-15% PAGE, transferred to nitrocellulose membrane and incubated with the antisera. Molecular weight markers are presented in kilodaltons. Antisera diluted 1:100.

Agglutination experiments

Intact *T. brucei* parasites were mixed in a 1:1 ratio (v/v) with sera of vaccinated and protected rats, vaccinated and non protected rats, or nonimmune rat serum. A sample was put on a glass slide and observed under a light microscope. Results show that while nonimmune sera, or sera from non protected animals had no effect on the parasites (Fig. 25A), the sera from the protected animals caused an immediate agglutination of the parasites (Fig. 25B). The higher the antibody titer, the more apparent the agglutination was. Agglutination of parasites was also observed with sera from protected animals which were heat inactivated for 30 min at 56°C.

Figure 25. A differential agglutinating capacity of sera from non protected (A) and protected (B) rats. Parasites were incubated in the presence of sera of vaccinated and non protected (A, x700) or protected (B, x700) animals, light microscopy.

A

B

Figure 25

Internalization of antibodies

Animals were vaccinated with the phosphocellulose purified proteins which are intracellular antigens, i.e. microtubular and glycosomal proteins. In order to exclude the possiblity that agglutination occurred because of recognition and crosslinking of external antigens, we incubated the parasites with sera of protected rats (1:1 ratio v/v) for 1 min (Fig. 26B) and for 15 min (Fig. 26C) at 22°C, washed the parasites from the free serum by centrifugation, fixed them in 0.1% glutaraldehyde, cryosectioned them and antibody was detected using gold conjugated anti rat antibodies. For the sake of comparison, we incubated parasites in serum which was raised against whole trypanosomes and which recognized the surface coat of the trypanosome (S. Himmelhoch, personal communication) (Fig. 26A), and which also caused agglutination of the parasites (not shown). Results show that while antibodies were found on the surface of the parasites when incubated in the presence of the serum which was raised against whole trypanosomes (Fig. 26A), no antibodies were detected on the surface of the parasites which were incubated with the sera from protected animals. Antibodies were detected at first within the flagellar pocket (Fig. 26B) and then inside the parasites (Fig. 26C). Without preincubation of the parasites with the sera, gold conjugated anti rat antibodies showed no labeling at all.

Figure 26. Internalization of antibodies: Parasites were incubated at 22°C with sera of rats exposed to a trypanosome infection (A-15 min. incubation, bar, 230nm), or with sera of vaccinated and protected rats (B-1min, C-15 min, bar, 260nm). Parasites were washed and fixed, cryosectioned and immunolabeled with 10nm gold conjugated anti rat antibodies. fl-flagellar pocket, f-flagella, gl-glycosome, sb-subpellicular microtubules, arrow indicating point of agglutination.

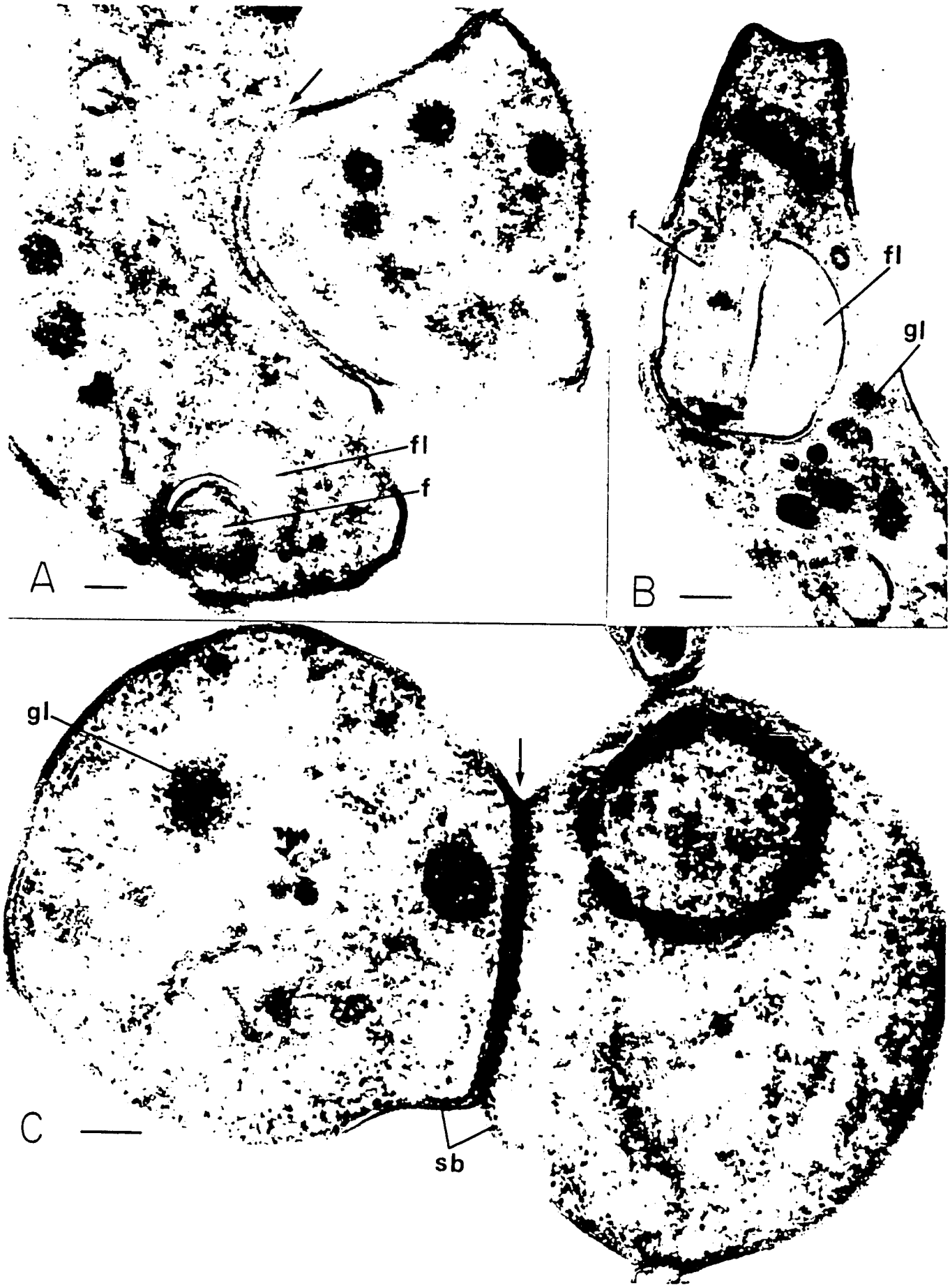


Figure 26

DISCUSSION

The aim of work presented in this chapter was to explore the possibility of using intracellular, stable, structurally important trypanosome microtubule components as protection-inducing antigens for vaccination against trypanosomiasis. The high degree of stability of the trypanosome subpellicular microtubules contrasts with that of many other eukaryotic cells (Stieger *et al.*, 1984). Since trypanosomal tubulin is rather similar to mammalian tubulin (Gallo & Precigout, 1988), the unique stability and structure desired in a potential vaccine should be sought in other trypanosome microtubule components. Microtubule associated proteins may be good candidates for this function.

We have isolated a protein fraction from *T. brucei* subpellicular microtubules containing 52kDa, 41kDa and 36kDa proteins which were characterized as a microtubule associated protein p52 (52kDa) and the glycosomal enzymes aldolase (41kDa) and GAPDH (36kDa).

When animals were vaccinated by the use of these trypanosomal proteins, 100% protection was achieved upon challenging the animals with *T. brucei*. Furthermore, since the hyperimmune anti *T. brucei* antigen sera were found to cross react with corresponding antigen preparations from *T. rhodesiense* and *T. evansi*, there is hope that the protective effect of vaccination with an antigen preparation from one trypanosome species will cross protect for different trypanosome species.

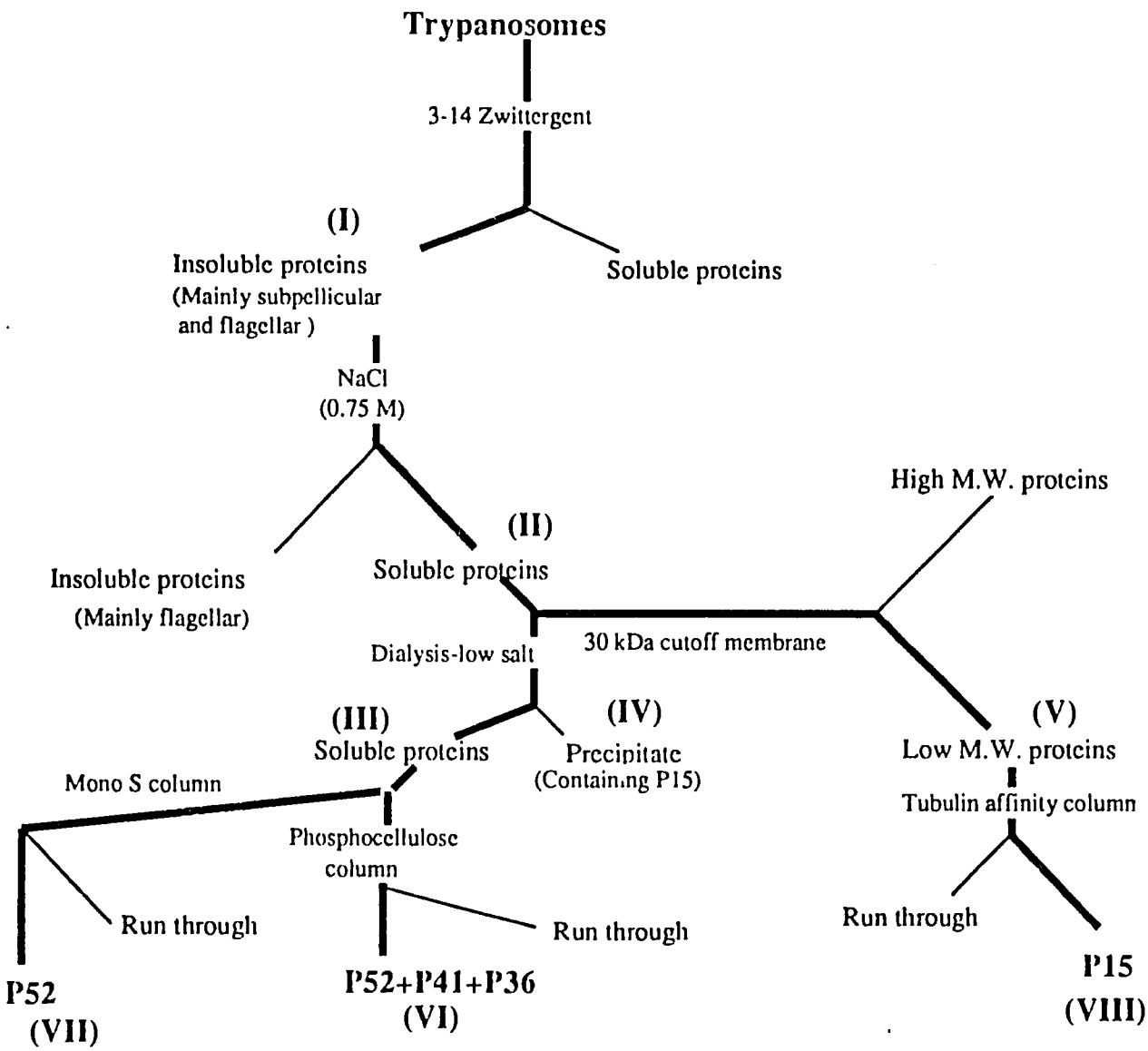
There is accumulating evidence that intracellular antigens can serve as protecting epitopes against parasitic diseases. For example, human resistance to *Schistosoma* has been found to be associated with IgG reactivity to a 37kDa protein (Dessein *et al.*, 1988) which was then identified as GAPDH (Dessein, personal communications). Paramyosin has been found to protect against schistosomes via a cell mediated mechanism (Sher *et al.*, 1989) and an intracellular leishmanial 10kDa protein was found to induce a T cell proliferation

response against *L. major* infection (Sher *et al.*, 1989). Furthermore, immunization with aldolase of *Plasmodium falciparum* induced immunity to malaria in monkeys (Certa *et al.*, 1988). The common notion is that these intracellular antigens are exposed at some point on the surface of the parasite or on the surface of the red blood cells in the case of malaria (Certa *et al.*, 1988). However in our work we show that when parasites were incubated with sera of vaccinated and protected rats, the parasites agglutinate. Within the detection limit, immunoelectron microscopy reveals no labeling on the surface of the parasites, but rather antibodies were detected within the parasite. We do not exclude the possibility that other factors than the antibodies in the sera of the vaccinated and protected animals caused agglutination. However the phenomenon of internalization of antibodies has been used for immunofluorescence studies in *Crithidia fasciculata* (Shlomai & Zadok, 1984), and uptake of macromolecules has been recorded (Opperdoes & Van Roy, 1982) where the molecules first accumulated in the flagellar pocket and then internalized via a mechanism of endocytosis or pinocytosis.

We do not know the nature of the protection we received and could not solve the mystery of its irreproducibility. Perhaps it is possible that the ratio between the injected proteins was important. Since the vaccination was made with structural proteins (p52) and functional proteins (aldolase and GAPDH), it may be possible that the right ratio between the two is important, taking into account the importance of the cytoskeleton to the integrity of the parasite and the importance of the glycosomal enzymes to its survival.

Appendix 1

Purification of Proteins P15 and P52



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Appendix 2

Amino acid analysis of p52

Amino acids	nmole
Glutamic acid	0.6664
Aspartic acid	0.4794
Alanine	0.4031
Leucine	0.3991
Proline	0.3837
Glycine	0.3568
Valine	0.3276
Arginine	0.3225
Phenylalanine	0.3215
Serine	0.2730
Threonine	0.1996
Lysine	0.1942
Isoleocine	0.1771
Tyrosine	0.1282
Cystein	0.08834
Methionine	0.06911

Amino acid analysis of p15

Amino acids	nmole
Alanine	0.2913
Tyrosine	0.1896
Glycine	0.1714
Glutamic acid	0.1478
Valine	0.1339
Lysine	0.1136
Aspartic acid	0.1022
Serine	0.09584
Threonine	0.08055
Leucine	0.05972
Isoleucine	0.01808

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