A high molecular mass phosphoprotein defined by a novel monoclonal antibody is closely associated with the intermicrotubule cross bridges in the Trypanosoma brucei cytoskeleton

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Summary

The main component of the cell body cytoskeleton of Trypanosoma brucei is the highly organised array of stable, subpellicular microtubules on the cytoplasmic face of the plasma membrane. Although several microtubule associated proteins (MAPs) have been shown to be associated with this array, the mechanisms by which individual microtubules interact with one another and with the membrane are still largely undetermined. In this study we have used the T. brucei cytoskeleton as a complex immunogen for the production of monoclonal antibodies to define novel cytoskeletal antigens. Screening by immunofluorescence enabled the selection of an antibody, WCB-1, which detects an antigen associated specifically with the subpellicular microtubules and not with the flagellum microtubules. The antigen (WCB210) was shown to have a relative molecular mass of 210 000 by western blotting. Immunogold studies showed the epitope to be located on the membrane-facing side of the subpellicular cage; it appears to be closely associated with the cross-bridges lying between the microtubules. Unlike many MAPs this protein was shown not to be heat stable and is predicted to be a roughly globular monomer. Even though WCB210 is a very minor component of the cytoskeleton it is heavily phosphorylated. It is possible that this protein is involved in regulation of the subpellicular microtubule crossbridges by interaction with other proteins.

Key words: trypanosome, cytoskeleton, microtubule associated protein (MAP), WCB-1.

Introduction

The African Trypanosoma brucei provides an ideal model for the investigation of microtubule-microtubule and microtubule-membrane interactions. T. brucei has a very stable cytoskeleton based around microtubules. Underlying the plasma membrane (pellicle) is a highly ordered array of microtubules, sometimes referred to as the subpellicular cage. These microtubules are orientated parallel to the long axis of the cell and form a helical array linked to the membrane. A second set of microtubules is confined to the flagellum. Here the microtubules of the axoneme in the typical 9+2 arrangement, are associated with the paraflagellar rod (De Souza and Souto-Padron, 1980), a highly ordered network composed mainly of two immunologically related proteins of apparent Mr 76 000 and 68 000 (Russell et al., 1983; Saborio et al., 1989). The cytoskeletal microtubules possess an unusually high degree of stability to cold and drug treatment, though biochemically trypanosome tubulin has been shown to be similar to mammalian brain tubulin (Steiger et al., 1984) and shows similar in vitro polymerisation characteristics (Dolan et al., 1986; MacRae and Gull, 1990). This may imply that the trypanosome microtubule associated proteins may be responsible for the apparent microtubule stability.

The microtubules of the subpellicular cage have been observed to have physical links with one another and with the overlying membrane (Vickerman, 1985; Jensen and Smail, 1986), yet little is still known about their molecular and structural organisation. Several proteins have been described which are candidates for MAP components of the subpellicular cage (reviewed by Robinson et al., 1991). Prominent among these candidate proteins are a family of high molecular mass, repetitive proteins, MARPs, which consist largely of a number of tandemly repeated, highly conserved 38-amino acid repeat units (Schneider et al., 1988). It has been shown that MARPs are located on the microtubules of the membrane skeleton and exclusively at
the membrane-orientated face (Hemphill et al., 1991). A recent study (Hemphill et al., 1992) shows that MARP1 represents a novel type of microtubule binding domain and binds to a site other than that used by MAP2 and Tau in tubulin binding. To date this is the most thoroughly characterised trypanosomal MAP and the most likely candidate for microtubule-microtubule or microtubule-membrane interactions.

Defining further MAPs is not straightforward. Due to the inherent stability of the subpellicular microtubules of Trypanosoma brucei and the problems involved in purification of low abundance proteins in the complex mixture of proteins in the cytoskeleton, conventional purification procedures are unlikely to be productive. In previous work we reported use of whole Trypanosoma brucei cytoskeletons as an immunogen for the generation of monoclonal antibodies which define specific spatial regions of the cytoskeleton (Woods et al., 1989). Here, a novel monoclonal antibody is described which has been generated using the same approach. The antigen is a high molecular mass phosphoprotein that colocalises specifically with the subpellicular microtubules and is therefore a candidate protein for involvement in microtubule cross-bridge function within the cytoskeleton.

Materials and methods

Trypanosomes

Procyclic Trypanosoma brucei, stock 427, were grown in tissue in tissue-culture flasks in semi-defined medium 79 (Brun and Schonenberger, 1979).

Fractionation of cells

Cytoskeletons containing the subpellicular cage, the flagellar microtubules and the parallaxerg rod were prepared according to the method of Woods et al. (1989).

The subpellicular microtubules were depolymerised and separated from the flagella using 1 M KCl as described by Woods et al. (1989). Briefly, isolated cytoskeletons were prepared from approximately 5 × 10^7 cells and incubated on ice for 40 min in PEmE (100 mM Na PIPES, 2 mM EGTA, 1 mM MgSO_4, 0.1 mM EDTA, pH 6.9), 1 M KCl in order to solubilise the subpellicular microtubules. The flagellum/flagellum attachment zone fraction was collected as a pellet by centrifugation at 11,600 g for 10 min.

Freeze/thaw lysis of cells. After harvesting by centrifugation, 10^7-10^8 cells were resuspended in 1 ml PBS (0.14 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, pH 7.4) containing protease inhibitors leupeptin (50 µg/ml), PMSF (50 µg/ml), pepstatin (5 µg/ml) and chymostatin (5 µg/ml), then quickly frozen in liquid nitrogen, thawed and vigorously mixed. The freeze/thaw cycle was repeated three times before centrifugation at 11,600 g for 10 min. The pellet consists of membranes and associated proteins and the supernatant contains cytosolic proteins that have spilt out of the lysed cell.

Proteins and antibodies

Bovine brain MAP2 was kindly donated by Dr Kate Treherne (University of Kent). The DR1 antibody (specific for the neuronal membrane skeletal protein A60) was generously donated by D. A. Rayner (Rayner and Baines, 1989); this antibody shows no cross-reaction with non-neuronal cells. TAT1 anti-tubulin antibody was produced in this laboratory and is described elsewhere (Woods et al., 1989). 5E9, an antibody specific for the parallaxerg rod proteins (Mr 76,000 and 68,000), was generously donated by J.-M. Gallo. Sheep brain tubulin was purified using phosphocellulose according to the method of Weingarten et al. (1974).

Production of monoclonal antibody, WCB-1

Mice were immunised with detergent-insoluble cytoskeletons and fusion of mouse splenocytes and Sp2/0Ag myeloma cells was carried out according to the method of Woods et al. (1989). Screening of positive wells was done by immunofluorescence on cytoskeletons, also in accordance with this method.

Immunogold electron microscopy

The method of Sherwin and Gull (1989) was used. Cells were harvested and washed in PBS. After settling on Formvar-filmed carbon-coated grids, they were then extracted using 0.5% Nonidet P40/PEME and protease inhibitors as above. Cytoskeletons were fixed in 3.7% formaldehyde for 10 min, followed by washing in 20 mM glycine in PBS. Grids were then blocked with 1% BSA (bovine serum albumin) in PBS before incubation in first antibody (diluted in 1% BSA in PBS) for 45-90 min at room temperature. After washing in 1% BSA in PBS (five washes each of 5 min) the grids were incubated in second antibody (rabbit anti-mouse 10 nm gold conjugate, Biocell) diluted 1:20 in 1% BSA on PBS for 45-90 min. Unbound material was removed by successive washes in the following: 1% BSA in PBS, 0.1% BSA in PBS, PBS alone (five washes of 5 min each solution). Cytoskeletons were then refluxed in 2.5% (v/v) glutaraldehyde in PEME for 30 s and stained using 2% ammonium molybdate, pH 7.0, with a drop of stain and removal using a suction pump so that liquid passed over the surface of the grid.

Specimen preparation for critical point drying

Fixed trypanosome cytoskeleton preparations were dehydrated through a graded series of acetone solutions followed by 3 changes of 30 min each in absolute acetone. The samples were then transferred to a Polaron critical point drier and dried by a standard schedule using liquid carbon dioxide.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was carried out by the method of Laemmli (1970) using SDS obtained from Sigma (Best et al., 1981). Polypeptides were either stained using Coomassie Brilliant Blue R250 or transferred to nitrocellulose paper (0.45 µm pore size, Schleicher and Schuell) as described by Towbin et al. (1979), and probed with antibodies as described by Woods et al. (1989) diluting antibody supernatants with 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.5% (v/v) Tween 20 as indicated in text and figure legends.

Two-dimensional gel electrophoresis

Two-dimensional isoelectric focusing-SDS gel electrophoresis was carried out according to the method of O’Farrel (1975), as modified by Birkett et al. (1985).

Gel filtration

The Stokes radius (R_s) of WCB210, the protein recognised by WCB-1, was determined by gel filtration on a Sephacryl S-300 (HR) (Pharmacia) gel filtration column (39 cm × 1 cm) equilibrated in PEME-1 M KCl and calibrated with thyroglobulin (Rs 8.6 nm), β-galactosidase (6.8 nm), catalase (5.2 nm), alcohol dehydrogenase (4.5 nm) and ovalbumin (3.6 nm).

Sucrose gradient sedimentation

The sedimentation coefficient of WCB210 was determined by centrifugation in 5-30% sucrose gradients made in PEME-1 M KCl.
and absence of 1.8 mg/ml sheep brain tubulin. After incubation for 8-72 h with intensification screens.

The cells were incubated at 28°C for 30 min the polymerised solution was either centrifuged to obtain a pellet from which samples were prepared for SDS-PAGE, or microtubules formed were settled onto electron microscopy grids and the immunogold procedure carried out.

Polymerisation of tubulin in the presence of WCB210

Soluble KCl extracts of cytoskeletons from 1.4 × 10^9 cells were desalted using Sephadex G25 (bead size 20-50 µm) swollen according to the manufacturer’s recommendation (Sigma) and equilibrated into PEME. A 6 cm × 1 cm column was packed and 1 ml of sample applied and eluted with PEME at 0.25 ml/min. To ensure the eluted protein was free of salt, 1% sodium dodecyl-sulphate (SDS) was added to a small volume of the sample and the absence of a potassium dodecylsulphate precipitate was checked. The desalted fraction was then incubated at 37°C in the presence of taxol (250 µM) and 1 mM GTP in both the presence and absence of 1.8 mg/ml sheep brain tubulin. After incubation for 30 min the polymerised solution was either centrifuged to obtain a pellet from which samples were prepared for SDS-PAGE, or microtubules formed were settled onto electron microscopy grids and the immunogold procedure carried out.

P32-labelling of Trypanosoma brucei

Cells (1.5 × 10^9) were harvested by centrifugation and washed in ME 83 media, (Seebeck and Kurath 1983), then suspended in 10 ml ME-83 containing 500 µCi disodium [32P]orthophosphate (ICN). The cells were incubated at 28°C for 2 h before centrifugation and washing twice in PBS. The cells were then incubated on ice for 15 min in 100 µl PHEME, 0.5% NP40, protease inhibitors as above, 0.2 mg/ml DNAase and 0.2 mg/ml RNAase before further fractionation or addition of gel sample buffer. For identification of 32P-labelled polypeptides by autoradiography, polypeptides were resolved on one- or two-dimensional gels. The gels were either stained with Coomassie Blue and dried onto Whatman 3MM paper or transferred to nitrocellulose paper for western blotting. The dried gels or probed blots were autoradiographed at ~80°C for 8-72 h with intensification screens.

Immunoprecipitation

The method used was adapted from that of Mose-Larson et al. (1982). Protein A-Sepharose was washed ×3 in TD buffer (1% v/v Triton-X 100, 1% deoxycholate, 150 mM Tris-HCl, 1 mM EDTA, pH 7.5) in the presence of 0.1% SDS (TDS). 100 µl of a 50% slurry was used for each precipitation and added to 5 ml of tissue culture supernatant of the desired antibody and mixed gently for 1 h. The Protein A-beads were then washed in TDS buffer and added to the protein sample as follows: 100 µl SDS gel sample buffer was added to the protein sample and heated to 100°C for 5 min. This was then cooled on ice before diluting 1:20 in TD buffer. To this were added 10 µl neutral mouse serum followed by mixing for 5 min before 3 additions of Protein A-Sepharose (prewashed ×3 in TDS buffer) spinning down the beads between additions. This should have cleared the sample of proteins that bind non-specifically to mouse proteins. The Protein A-beads conjugated to the mouse antibody were then added to the cleared trypanosome sample and incubated with gentle agitation for 1 h. The beads were separated and washed well in TDS buffer before being boiled in SDS sample buffer and loading onto gels.

NaOH stripping of trypanosome membranes

This was carried out by a method similar to that of Davis and Bennett (1986). Cells were washed in PBS and resuspended in distilled water (1 ml) containing pepstatin (5 µg/ml), chymostatin (5 µg/ml), leupeptin (50 µg/ml) and PMSF (50 µg/ml) and sonicated at high speed for 30 s in order to break up the cells. They were then centrifuged for 10 min at 11 500 g to harvest the membranes, which were then resuspended in 0.4 ml 0.1 M NaOH, or distilled water as a control. These were then left on ice for 1 h before centrifugation in an airfuge at 20 000 psi (80 000 g) in order to separate those proteins remaining with the membrane (in the pellet) from the peripheral proteins present in the supernatant. 1 M Tris, pH 2, was added to the supernatant in order to neutralise it and the pellet was gently washed in PBS before addition of sample buffer.

Extraction with Triton-X114

The method of Pryde and Phillips (1986) was used. Cells or soluble extracts were resuspended in 0.5 ml PHEME at 4°C and a 10% Triton-X114 stock solution was added to give a final Triton-X114 concentration of 2%. This was mixed thoroughly and left on ice for 15 min. Insoluble proteins were removed by centrifugation at 4°C at 11 500 g for 5 min in a microfuge. The supernatant was then warmed to 37°C for 15 min. The now cloudy solution was centrifuged for 5 min, 11 500 g in the warm. Both the top, aqueous layer and the bottom, lipophilic layer were prepared for SDS-PAGE by addition of gel sample buffer.

Protease treatment of whole cells

Cells were harvested by centrifugation and washed in PBS before resuspension in PBS containing 5 mM dithiothreitol (DTT), 0.5 mg/ml chymotrypsin, 0.5 mg/ml trypsin and 0.5 mg/ml papain. The cells were then incubated at 37°C for 1 h and washed ×3 in PBS and ×1 in PBS containing iodoacetamide (1 mg/ml), leupeptin (50 µg/ml), phenylmethylsulphonyl fluoride (PMSF) (50 µg/ml), pepstatin (5 µg/ml), chymostatin (5 µg/ml) and EGTA (1 mM).

Results

Production of the WCB-1 antibody and immunolocalisation of its antigen

We reasoned that it would be possible to define sub-pellicular proteins associated with microtubules by immunofluorescence screening of supernatants from a panel of hybridomas raised using whole cytoskeletons as an immunogen. A monoclonal antibody defining such a protein should stain in immunofluorescence only the cell body and not the flagellum. Hybridomas were prepared as described in Materials and methods. Hybridoma supernatants were screened by immunofluorescence on whole trypanosome cytoskeletons. This allowed isolation of a clone that secreted an antibody which gave the staining pattern shown in Fig. 1 (C and F). The stain is absent from
the flagellum (compare A and D with C and F), but a bright fluorescence is observed over the rest of the cell surface. This antibody was designated WCB-1 (whole cell body, to indicate its staining pattern). This staining pattern is distinct from tubulin or that of the panel of antibodies previously described. The WCB-1 pattern defines an antigen specifically associated with the subpellicular cage. The staining with WCB-1 does not alter during the cell cycle, which implies that the presence or spatial arrangement of the protein recognised by WCB-1 is not dependent on the position of the cell in the cell cycle (shown in Fig. 1).

To investigate the ultrastructural location of the WCB-1 antigen WCB210, immunogold staining of detergent-insoluble cytoskeletons was carried out (Fig. 2). Fig. 2A confirms that the epitope is not located in the flagellum but colocalizes with the subpellicular microtubules only. Comparison of WCB-1 staining patterns with those of an anti-tubulin antibody, TAT1, shows that the WCB-1 label is far less dense than the TAT1 (Fig. 2B). This is not surprising as tubulin is by far the most abundant protein in the subpellicular cage. A further immunogold study was carried out where the cytoskeletons were critically point dried after labelling and staining, allowing them to retain their 3-dimensional structure. Labelling of such cytoskeletons is

![Images of immunofluorescence staining patterns](image-url)
shown in Fig. 3A and B. Labelling with WCB-1 results in the gold particles lying to the side of the microtubules rather than labelling them directly (shown in Fig. 3A,B). It is however, difficult to determine whether WCB-1 is actually binding to the microtubule cross-links, as it is difficult to distinguish between the protein cross-links and the antibody-protein complex. Where the cytoskeletons have become slightly disrupted it can be seen that the antibody labels a single surface of the microtubules only. The WCB-1 antibody preferentially stains the exterior side of the subpellicular microtubules, that side nearest the plasma membrane (Fig. 3A). This was not found to be the case for similarly treated cytoskeletons labelled with the anti-tubulin TAT1, where the gold particles were distributed fairly evenly on both surfaces of the subpellicular microtubules.

When whole unfixed or fixed cells were incubated with WCB-1 followed by gold-conjugated second antibody, no specific label of protein was seen (not shown). This implies that the epitope recognised by WCB-1 is not exposed on the external surface of the cell.

**WCB-1 recognises a polypeptide of $M_r$ 210 000**

The nature of the antigen was analysed using western blotting (Fig. 4). WCB-1 recognised polypeptide(s) of $M_r$ 210 000 ± 10 000. The stained polypeptide band appears rather broad, especially when run on long (14 cm) gels. The apparent $M_r$ seems to drop in samples prepared in the presence of detergent (see Fig. 4B lanes 2 and 6 compared to lane 1). This drop in size is not prevented by the presence of protease inhibitors (leupeptin, 50 µg/ml; PMSF, 50 µg/ml; chymostatin, 5 µg/ml and pepstatin, 5 µg/ml). To indicate its size, the antigen was designated WCB210.

**Subcellular and species distribution of WCB210**

WCB210 is not solubilised by detergent (Fig. 4B lane 2). When cells are lysed by freezing and thawing (see Materials and methods), WCB210 remains with the membranous fraction (Fig. 4B, lane 3) and is absent from the cytosolic fraction (Fig. 4B, lane 4). Extraction of cytoskeletons with 1 M KCl solubilises WCB210 along with the subpellicular microtubules (Fig. 4B, lane 5), and none remains with the flagellum (Fig. 4B, lane 6). These data are consistent with the immunofluorescence observations. The protein band recognised by WCB-1 on western blots is not visualised on gels by either Coomassie Blue or silver staining, suggesting that WCB210 is a protein of low abundance. This is consistent with the immunoelectron microscopy comparison of the abundance of tubulin and WCB-1 antigens.

WCB-1 did not detect any proteins from samples of the vertebrate PtK2 cells, or other trypanosomatids such as *Crithidia fasciculata*, bloodstream *T. lewisi* or bloodstream *T. congolense* on blots; nor did it detect brain antigens by immunoperoxidase staining of sections of rat brain.
**WCB210 is a peripheral membrane protein**

Since WCB210 is close to the plasma membrane, it was important to determine whether it was integral to the membrane, or whether it behaved as a peripheral membrane protein. Proteins separated into hydrophilic and hydrophobic

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**Fig. 3.** Immunogold labelling of a critically point dried, positively stained cytoskeleton using WCB-1. The cytoskeleton is preserved on the grid in a way that reflects the native structure. In A the microtubules lying underneath are in contact with the grid, and so their cytoplasmic face can be seen; the microtubules lying on top are folded over so that the membrane-facing surface is exposed. Note that gold lies to the side of microtubules, coincident with cross-bridges, and preferentially labels one face of the microtubule array, corresponding to the side closest to the plasma membrane. B shows an enlarged area of A.
fractions by Triton X-114 extraction and temperature-dependent phase separation were blotted and probed with WCB-1. WCB210 separated into the aqueous (hydrophilic) phase (not shown); this indicated that WCB210 was hydrophilic, and unlikely to be integral to the plasma membrane.

A property of integral membrane proteins is that they remain with the membrane after treatment with alkali solutions, which solubilises peripheral proteins from the membrane. Treatment of sonicated trypanosome cell membranes with 0.1 M NaOH left no proteins detectable by Coomassie Blue staining associated with the membrane. WCB210 was solubilised from membranes by 0.1 M NaOH and was detected by western blotting the NaOH supernatant (not shown).

Treatment of whole cells with a mixture of chymotrypsin, trypsin and papain, as detailed in Materials and methods, did not result in a drop in the apparent molecular mass of WCB210 on western blots (not shown). This shows that either part of the protein is not exposed on the exterior of the cell for access to proteases, or that there is no sequence suitable for cleavage by the mixture of proteases used.

All these criteria indicate that WCB210 is not transmembrane, and is confined to the cytoplasmic face of the membrane.

**WCB210 is heat labile**

High $M_r$ MAPs are sometimes heat stable. WCB210 was tested for its heat stability (not shown). A 1 M KCl extract of cytoskeletons was heated to 80°C for 5 min and centrifuged to pellet any proteins denatured by heating. WCB210 pelleted along with other aggregated, denatured proteins. On the other hand, MAP2 treated in parallel remained unpelleted. Thus, WCB210 does not appear to be heat stable.

**Investigation of a possible interaction of WCB210 with tubulin in vitro**

To determine whether WCB210 could bind to polymerised tubulin, cytoskeletal proteins (solubilised from cytoskeletons with 1 M KCl) were desalted into PEME/GTP, and were incubated in the presence and absence of brain or trypanosome tubulin under polymerising conditions (see Materials and methods). After incubation, the microtubules were pelleted. Samples of supernatants and pellets were analysed by SDS-PAGE and western blotting using WCB-1. WCB210 appeared to pellet along with both brain and trypanosome tubulin (not shown). However, it could not be demonstrated by electron microscopy that WCB210 was bound to microtubules in these experiments. When incubation mixtures were analysed by immunogold and negative stain electron microscopy, no specific labelling of any structure, including microtubules, was observed. Firm conclusions as to whether WCB210 bound to microtubules cannot be made from this experiment, although WCB210 showed no tendency to pellet in the ultracentrifuge unless polymerised tubulin was present.

**Physical properties of WCB210**

Blots of 2-D gels of salt-extracted proteins from cytoskeletons probed with WCB-1 give a staining pattern of two spots, which run directly above $\beta$-tubulin, and have a calculated pI value of 5.6-6.2 (not shown).

An estimate of the sedimentation coefficient was obtained by sucrose gradient centrifugation of KCl extracts of cytoskeletons. Linear sucrose gradients (5-30% in PEME/1 M KCl) were calibrated with a series of standards of known $s$ value. In these gradients, WCB210 sedimented at 10.4 ± 0.2 S. For comparison, trypanosome tubulin dimers sedimented at 5.85 ± 0.2 S.

To determine the Stokes radius ($R_s$) of WCB210, KCl extracts were chromatographed on a calibrated Sephacryl S300HR column. The $R_s$ value of WCB210 was determined to be 5.9 ± 0.3 nm.

Using equations (1) and (2) described in Materials and methods, the data indicates that the $M_r$ of WCB210 is 248 000 (± estimated 20 000). The frictional ratio is between 1.2 (using $\delta = 0.4$) and 1.05 (using $\delta = 1$). A frictional ratio of 1.05-1.2 implies that WCB210 is roughly globular (a value of 1 would imply complete symmetry).

The values for $M_r$ obtained by SDS-PAGE and by analysis of physical properties are in good agreement. Within the limits of error, the values for $M_r$ and frictional ratio imply that WCB210 is a roughly globular monomer. However, it is conceivable that the discrepancy between the $M_r$ values obtained by the two different methods may reflect the asso-
The observation that WCB210 represents a globular monomeric protein which does not necessarily bind tubulin directly, raised the question of whether or not it might be a regulatory protein associated with the cytoskeleton. If so, it might be regulated by covalent modification. To test this, the possibility that it might be phosphorylated was examined. Trypanosomes were cultured in the presence of $^{32}$P orthophosphate. Coomassie Blue stained SDS-PAGE of the resulting $^{32}$P-labelled cells and extracts are shown in Fig. 5A; corresponding autoradiographs are shown in Fig. 5B. Many proteins are heavily labelled (Fig. 5B, lane 1); some of these proteins are solubilised by detergent extraction (Fig. 5B, lane 3) though at least 9 of these bands are labelled polypeptides retained by the cytoskeleton (Fig. 5B, lane 2). Most of the $^{32}$P-labelled polypeptides remain with the membrane fraction after freeze/thaw lysis (Fig. 5B, lane 4) though one major phosphorylated polypeptide ($M_r$ approx. 190 000) appears to be cytosolic (Fig. 5B, lane 5). There is little label in the flagellar fraction (lane 6) though at least 6 phosphorylated polypeptides are solubilised by 1 M KCl (Fig. 5B, lane 7) and therefore associated with the subpellicular cage. A highly labelled polypeptide appeared at $M_t$ 210 000. Other phosphorylated protein bands in the KCl extract were of $M_t$ 68 000, 85 000, 100 000, 120 000 and 135 000. The 210 000 band has the same distribution throughout the extracts as WCB210, i.e. present in whole cells, cytoskeletons, freeze/thaw membranous fraction and KCl extract, but absent from the detergent soluble fraction, the freeze/thaw cytosolic fraction and the KCl flagellar fraction.

To determine whether the $M_t$ 210 000 protein that incorporated $^{32}$P is WCB210, a 2-D gel of the labelled cytoskeletons was blotted and probed with WCB-1 and autoradiographed briefly to reveal the most heavily $^{32}$P-labelled proteins, which should have emphasised the $M_t$ 210 000 protein. The labelled spot on the autoradiograph at $M_t$ 210 000 (see Fig. 6) coincided exactly with the stained spot on a blot probed with WCB-1, confirming that WCB210 is phosphorylated. The second major spot visualised on the autoradiograph of the 2-D blot was that of the $M_t$ 68 000 labelled protein. It was possible that this represented the $M_t$ 68 000 PFR protein. However, probing the blot with 5E9 (see Fig. 6C), an anti-PFR antibody (kindly donated by J.-M. Gallo), showed that the labelled protein was distinct from the PFR protein, which is more acidic than PFR.

Further conformation that the phosphorylated $M_t$ 210 000 protein was indeed WCB210 was obtained by immunoprecipitation of the $M_t$ 210 000 radiolabelled protein by WCB-1. This is shown in Fig. 7. The precipitation of a $M_t$ 210 000 phosphoprotein by WCB-1 was shown to be specific, as an unrelated hybridoma supernatant, DR1, (an antibody specific for the neuronal cytoskeleton protein, A60; Rayner and Baines, 1989), did not precipitate this antigen. Note that the heavy bands precipitated at the top of the autoradiograph in both DR1 and WCB-1 samples represent non-specific precipitation of (probably) aggregated material, which does not enter the resolving gel.

It was noted in the original characterisation of WCB210 that it ran as a broad band on SDS-PAGE. Since the protein is phosphorylated, it was possible that phosphorylation

<table>
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<th>Property</th>
<th>Value</th>
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<tr>
<td>Sedimentation coefficient</td>
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<tr>
<td>Stokes radius</td>
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<td>Isoelectric point</td>
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<tr>
<td>Calculated $M_r$ in crude KCl extract</td>
<td>248 000 ± 20 000</td>
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<tr>
<td>$M_t$ (SDS gel electrophoresis)</td>
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Table 1. Properties of WCB210

Fig. 5. $^{32}$P labelling of trypanosomal proteins. A shows a Coomassie Blue-stained gel and B represents the corresponding autoradiograph. Lane 1, whole cells; lane 2, detergent-insoluble cytoskeletons; lane 3, detergent-soluble fraction; lane 4, freeze/thaw membrane fraction; lane 5, freeze/thaw cytosolic fraction; lane 6, KCl flagellar fraction; lane 7, KCl subpellicular fraction. W marks the position of the protein suspected to be WCB210.
altered the mobility of the protein on gels, especially since
\( ^{32} P \)-labelled WCB210 in immunoprecipitates ran as a very
broad band: precedents exist for this in the case of the
neurofilament H and M subunits (Carden et al., 1985).
Treatment of trypanosome cytoskeletons with alkaline
phosphatase, however, did not result in an alteration of the
apparent molecular mass of WCB210.

**Discussion**

The biochemical identification of trypanosomal MAPs is
problematic due to their low abundance and the inherent
stability of the subpellicular microtubules. Thus, we have
adopted the approach of producing a probe in the form of
a monoclonal antibody produced against an antigenic deter-
minant present in a complex mixture of proteins, i.e. the
cytoskeleton itself. Screening by immunofluorescence
enabled the selection of an antibody that associated with
the subpellicular microtubules and not those of the flagel-
rum. On the premise that any protein involved in subpel-
licular microtubule organisation would have such a cell-
ular distribution, the protein recognised by the antibody
could be characterised further. This method of screening
also permitted detection of very small signals which may
be present in only a minor component of the whole struc-
ture and may go undetected by other methods.

The fact that WCB210 colocalises with the subpellicular
microtubules and not the flagellum (as determined by
immunoelectron microscopy) makes it a strong candidate
for a protein involved in subpellicular microtubule func-
tion. The most likely role for WCB210 is probably related
to the intermicrotubule cross-links. Evidence for this comes
from immunoelectron microscopy which indicates that
WCB210 is very closely associated with the cross-links.
Whether it is a cross-linking protein *per se* is unclear: its
globular shape would be atypical for a cross-linking pro-
tein, and no firm electron microscopic evidence could be
obtained for a direct interaction of WCB210 with tubulin
in reconstitution experiments *in vitro*. An alternative pos-
sible role is suggested by the observation that the epitope
recognised by WCB-1 appears to be located on the mem-
brane-bound face of the subpellicular cage. This may
suggest a role in membrane binding, though no other evi-
dence to this effect has been obtained. In this context it
should be noted that no evidence was obtained for a trans-
membrane or integral characteristic of the protein (the epi-
tope is not exposed on the cell surface, and WCB210 can
be solubilised from membranes with NaOH), neither did it
seem to be sufficiently amphitropic to be a likely linker.
globular MAPs do exist, for example, the sea urchin egg and WCB-1 represents non-specific precipitation of material proteins from whole cells, and lane 4 from whole cytoskeletons. MAP, buttonin that decorates the spindle microtubules between microtubules and the lipid bilayer (it is soluble in aqueous solutions and does not interact with Triton X-114).

Nonetheless, the possibility that WCB210 links the subpellicular microtubules to the plasma membrane indirectly via other proteins, cannot be excluded.

The predicted shape of WCB210, a globular monomer, is uncharacteristic of many MAPs. In terms of molecular structure, MAP1A and MAP2 have been shown to consist of arm-like projections from the microtubule surface (Kim et al., 1979; Shiomura and Hirokawa, 1987). Rotary shadowing revealed that they both take the shape of long thin structures (Voter and Erickson, 1982; Gottlieb and Murphy, 1985). Tau has also been shown to be a long and flexible molecule (Mandelkow and Mandelkow, 1979). Thus, many MAPs appear to be elongated molecules. On the other hand, globular MAPs do exist, for example, the sea urchin egg MAP, buttonin that decorates the spindle microtubules (Hirokawa and Hisanaga, 1987).

Incorporation of $^{32}$P into WCB210 shows it to be a phosphoprotein. This is a very common characteristic of MAPs. Tau, MAP2 and STOP proteins are all known to be reversibly phosphorylated. The smeared appearance of WCB210 on blots may imply several states of phosphorylation. Alkaline phosphatase does not alter the apparent phosphorylation regulating cross-link formation can be taken as it does for neurofilament heavy chain polypeptide.

References


Fig. 7. Immunoprecipitation of $^{32}$P-labelled proteins with WCB-1. The figure shows an autoradiograph of an SDS gel of samples obtained from $^{32}$P-labelled cells (lanes 1-3) and cytoskeletons (lanes 4-6). Lane 1 shows SDS-solubilised labelled proteins from whole cells, and lane 4 from whole cytoskeletons. Lanes 2 and 5 represent the (non-specific) immunoprecipitation due to DR1 and lanes 1 and 6, represent the immunoprecipitation due to WCB-1. Note that the material precipitated by both DR1 and WCB-1 represents non-specific precipitation of material which does not enter the resolving gel. WCB210 is visualised as a broad band in lanes 3 and 6.


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