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A 60-kDa cytoskeletal protein from *Trypanosoma brucei brucei* can interact with membranes and with microtubules

(parasitic protozoa/microtubule-associated proteins/Trypanosomatidae/liposomes)

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ABSTRACT **The cytoskeleton of eukaryotic cells is a major determinant of cellular architecture and of many cellular functions. In addition to or in place of the transcellular cytoskeleton, many eukaryotic cells also contain membrane-associated cytoskeletal structures (membrane skeletons), which are important for cellular structure and function. The membrane skeleton of the parasitic hemoflagellate *Trypanosoma brucei* consists of a dense array of singlet microtubules (subpellicular microtubules), which are tightly associated to the overlying cell membrane. This study reports the identification of a microtubule-associated protein from *Trypanosoma brucei* that constitutes a component of the link between this microtubular array and the cell membrane. The protein can bind *in vitro* both to microtubules and to membrane vesicles or liposomes. Furthermore, it can crosslink microtubules and membrane vesicles, suggesting that it exerts a similar function in the membrane skeleton.**

Cytoskeletal structures that are intimately associated with the plasma membrane and that constitute essential functional components of the membrane have been identified in numerous eukaryotic cells. The membrane skeleton that has been studied in most detail is the spectrin network of the human erythrocyte (1–4). However, investigations of the membrane skeletons in many other cell types have revealed a wide variety in their composition and structure (5–9). In the parasitic hemoflagellate *Trypanosoma brucei brucei*, the entire plasma membrane is underpinned by a regular array of singlet microtubules (9–14). This microtubular membrane skeleton forms the main structural component of the cell body. No transcellular cytoskeletal structures have yet been identified, suggesting that the microtubule-based membrane skeleton is the prime determinant of the cellular architecture. Very little is known of how these microtubules are connected to the overlying cell membrane. Recent studies have shown that the microtubules of the trypanosomal membrane skeleton are composed of similar tubulin isotypes as are, for example, those of the flagellar axoneme (9, 15). Analyses of the trypanosomal tubulin genes and their transcripts have also provided strong evidence that the multiple tubulin genes of *T. brucei* in fact give rise to only one isotype each of α-tubulin and of β-tubulin (9, 16–19). Thus, a direct interaction of the subpellicular microtubules with the overlying cell membrane via a particular membrane-specific tubulin isotype (20) or via a posttranslational modification of the tubulins (21) seems unlikely for the trypanosomal membrane skeleton.

Consequently, microtubule-associated proteins may be required to mediate the contact between the microtubular array and the cell membrane. The contact between the microtubular array and the membrane can be disrupted *in vivo* by the action of chlorpromazine and related phenothiazines (22). A 60-kDa protein (p60) has been isolated from trypanosomes by chlorpromazine affinity chromatography, and monoclonal antibodies against it have been described (23). Recently, a 61-kDa protein that may be a homologue of p60 has been identified in the cytoskeleton of a related trypanosomatid, *Crithidia fasciculata* (24).

The present study demonstrates the ability of the p60 protein from *Trypanosoma brucei* to interact both with microtubules and with membrane vesicles or liposomes *in vitro*. Furthermore, evidence is presented that it can crosslink microtubules and membrane vesicles, suggesting that this protein may indeed function as a component of the microtubule/membrane link in the trypanosomal membrane skeleton.

MATERIALS AND METHODS

Copolymerization Assay. Cytoskeletons were prepared from cultured procyclic *Trypanosoma brucei brucei* stock STIB 366 as described (9), suspended to 5×10^8 per ml in Mops/$MgCl_2$/EGTA buffer (10 mM Mops/1 mM $MgCl_2$/0.1 mM EGTA, pH 6.9), and sonicated five times for 30 sec at an amplitude of 8 μm with an MSE Soniprep 150 sonifier (MSE Scientific Instruments, Crawley, Sussex, England) equipped with a microtip. Particulate components were sedimented in a Beckman Ti 50 rotor at 35,000 rpm (110,000 $\times$ *g*) for 45 min at 2°C. Microtubules were polymerized from the high-speed supernatant by addition of taxol to 20 μg/ml and GTP to 1 mM, followed by a 15-min incubation at 37°C (25). After quenching in ice, microtubules were collected by sedimentation through a cushion of 20% (wt/vol) sucrose in Mops/$MgCl_2$/EGTA buffer containing taxol and GTP, in a Ti 50 rotor at 35,000 rpm for 30 min at 2°C.

Microtubule Binding Assay. Microtubules were prepared from cultured trypanosomes by a slight modification (V.K. and T.S., unpublished data) of the taxol procedure (25). For the binding assay, microtubules and p60 were incubated for 10 min at room temperature in a total volume of 180 μl in Mops/$MgCl_2$/EGTA buffer containing taxol (10 μg/ml) plus the additions specified in the text. A 50-μl cushion of 20% sucrose was underlayered, and microtubules were sedimented by centrifugation in a Beckman Airfuge for 15 min at 28 psi (170,000 $\times$ *g*; 1 psi = 6.89 kPa). Control experiments were performed to exclude the possibility that p60 sediments on its own under the assay conditions.

Liposome Binding Assay. Unilamellar liposomes were prepared by sonication of a 20-mg/ml suspension of lipid in 10 mM Hepes/100 mM sucrose/50 mM pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium), pH 7.2. Liposomes were purified by gel filtration through Sephadex G-25 (Pharmacia). p60 and liposomes were incubated for 15 min at room temperature in aliquots of 200 μl in Mops/$MgCl_2$/

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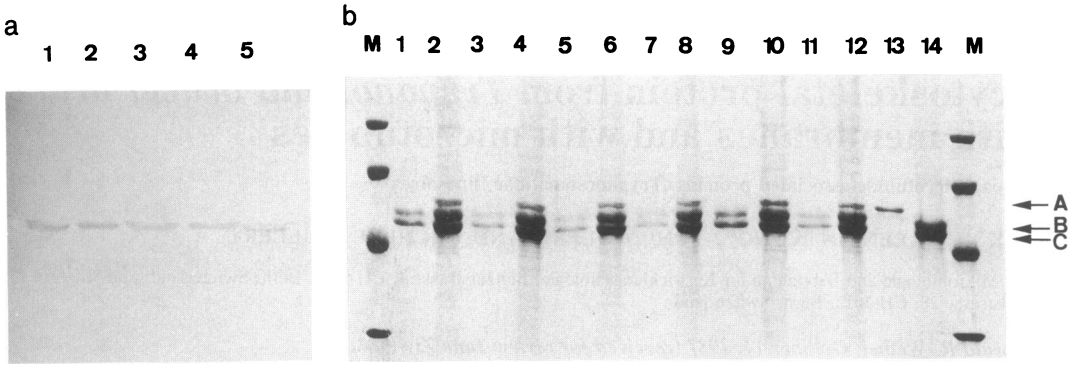

FIG. 1. (*a*) p60 copolymerizes with tubulin. Microtubules were polymerized from a whole cytoskeletal lysate. Equivalent aliquots of the total sonicate (lane 2), the high-speed supernatant before polymerization (lane 3), and the sedimented microtubules (lane 4) were analyzed by gel electrophoresis. Lanes 1 and 5 contained p60 alone as marker. p60 was detected by immunostaining with the monoclonal antibody 2D G3. (*b*) p60 binds to preformed microtubules *in vitro*. Salt-washed microtubules and p60 were incubated for 15 min at room temperature in a total volume of 180 μl in Mops/$MgCl_2$/EGTA buffer containing taxol (10 μg/ml) plus the additions specified below. p60 binding was subsequently analyzed as described in *Materials and Methods*. Pellets (even-numbered lanes) and supernatants (odd-numbered lanes) were analyzed by electrophoresis in an 8–15% acrylamide gel and stained with Coomassie blue. Lanes: 1 and 2, control (no additions); 3 and 4, 1 mM ATP; 5 and 6, 1 mM GTP; 7 and 8, 0.1% Triton X-100; 9 and 10, 1 mM $CaCl_2$; 11 and 12, 1 mM chlorpromazine; 13, p60 input; 14, microtubule input; M, molecular weight markers (carbonic anhydrase, M_r 31,000; ovalbumin, M_r 42,700; bovine serum albumin, M_r 66,200; phosphorylase *b*, M_r 97,400). Arrowheads A, B, and C designate the position of p60, α-tubulin, and β-tubulin, respectively.

EGTA buffer. The liposomes were then pelleted by centrifugation in a Beckman Airfuge for 30 min at 28 psi. Pellets and supernatants were analyzed by electrophoresis in 8–15% acrylamide gels, followed by transfer of the proteins to nitrocellulose and immunostaining.

p60 Preparation. p60 was isolated from trypanosomal cytoskeletons by a procedure to be detailed elsewhere. In brief, cytoskeletons were prepared as described (9), and p60 was extracted from the cytoskeletons and purified by a succession of gel filtration [Bio-Gel P100 (Bio-Rad) or Sephacryl 300 (Pharmacia)] and ion-exchange chromatography [DEAE-Sephacel (Pharmacia)]. Alternatively, p60 was purified from the crude cytoskeletal extracts by velocity sedimentation in glycerol gradients. Either procedure results in preparations of p60 that still contain additional minor bands when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Different contaminating bands are obtained in the different purification schemes. However, the results of the assays reported are consistent between p60 preparations obtained by different procedures and hence are not influenced by the contaminating proteins.

Antibody. The anti-p60 monoclonal antibody 2D G3 has been described (23).

RESULTS

p60 Copolymerizes with Tubulin. To establish whether p60 can interact with microtubules *in vitro*, we investigated its copolymerization with tubulin. Tubulin was polymerized from a high-speed supernatant of sonicated cytoskeletons in the presence of taxol and GTP, and the resulting microtubules were collected by centrifugation. When the resulting microtubules were analyzed by gel electrophoresis and immunoblotting with a monoclonal antibody against p60 (2D G3; ref. 23), p60 was found to cosediment with the microtubules formed in the reaction (Fig. 1*a*). The ability of p60 to copolymerize with tubulin suggests that it is indeed a microtubule-associated protein.

p60 Binds to Preformed Microtubules. As a next step, the interaction of p60 with preformed microtubules was tested under various conditions (Fig. 1*b*). Microtubules were prepared by taxol-induced polymerization (25) from whole trypanosome lysates and were salt-washed to remove all endogenous microtubule-associated proteins. p60 readily bound to such microtubules in the standard reaction buffer (Fig. 1*b*, lanes 1 and 2). This binding was not affected by the presence of 1 mM ATP (lanes 3 and 4), 1 mM GTP (lanes 5 and 6), 0.1% Triton X-100 (lanes 7 and 8), 1 mM $CaCl_2$ (lanes 9 and 10), or 1 mM chlorpromazine (lanes 11 and 12). Analysis of the p60 input and the microtubule input confirmed that p60 does not pellet on its own under the conditions of the assay (lane 13) and that microtubules prepared as outlined above are free of endogenous p60 (lane 14). This latter point has been further ascertained by immunoblotting experiments. The observation that binding was not affected by Triton X-100 confirmed that p60 does indeed bind to the microtubules, and not to membrane vesicles that are present in the microtubule preparations (refs. 25 and 26; see below). During the binding reaction in 1 mM $CaCl_2$ (lanes 9 and 10), a considerable amount of tubulin was solubilized from the microtubules. Nevertheless, p60 bound quantitatively to the remaining microtubules, indicating that soluble tubulin does not compete with microtubules for binding of p60. A similar series of binding experiments in the presence of increasing concentrations of NaCl showed that the binding of p60 to microtubules is sensitive to increased ionic strength, with no binding detectable at 250 mM NaCl (data not shown). This ionic strength dependence of the *in vitro* binding of p60 to microtubules closely parallels the salt requirements for the elution of the endogeneous p60 from

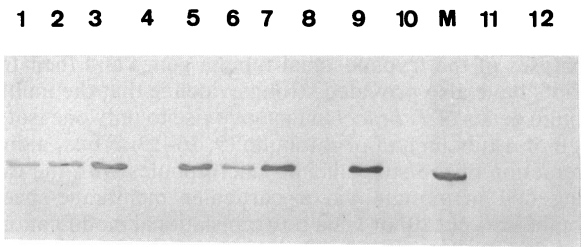

FIG. 2. p60 interacts with liposomes. p60 was incubated with asolectin liposomes in Mops/$MgCl_2$/EGTA buffer containing the additions indicated below. After sedimentation of the liposomes, pellets (even-numbered lanes) and supernatants (odd-numbered lanes) were electrophoresed in 8–15% acrylamide gels, followed by transfer of the proteins to nitrocellulose and immunostaining with the 2D G3 antibody. Lanes: 1 and 2, no additions (control); 3 and 4, 300 mM NaCl; 5 and 6, 1 mM ethanolamine; 7 and 8, 0.1% Triton X-100; 9 and 10, 1 mM chlorpromazine; 11 and 12, liposomes without added p60; M, p60 marker.

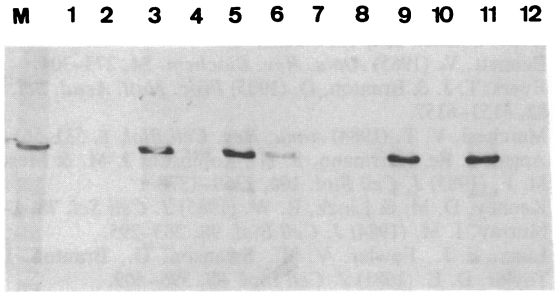

FIG. 3. Liposome binding of p60 is not indiscriminate. p60 was incubated with liposomes of defined composition in Mops/$MgCl_2$/EGTA buffer with or without 300 mM NaCl. p60 binding was then analyzed as outlined in the legend of Fig. 2. Lanes 1–6: phosphatidylcholine liposomes. Lanes 7–12: liposomes consisting of 70% (wt/wt) phosphatidylcholine and 30% phosphatidylethanolamine. Lanes 1, 2, 7, and 8: liposomes without added p60. Lanes 3, 4, 9, and 10: liposomes and p60 in buffer. Lanes 5, 6, 11, and 12: liposomes and p60 in buffer plus 300 mM NaCl. Lane M: p60 marker. Odd-numbered lanes: supernatants. Even-numbered lanes: pellets.

trypanosomal cytoskeletons. The data indicate that p60 behaves as a bona fide microtubule-associated protein (27) whose association with microtubules is similarly sensitive to ionic strength *in vivo* and *in vitro*.

p60 Binds to Synthetic Liposomes. The strong retention of p60 on hydrophobic chromatography matrices (ref. 23; V.K. and T.S., unpublished data) suggested the presence of a strongly hydrophobic domain in the molecule. This observation raised the possibility that p60 may be able to interact not only with microtubules but also with membranes. To explore this, we incubated p60 with synthetic liposomes prepared from soybean asolectin as a model membrane system. Liposomes were sedimented by ultracentrifugation, and the presence of p60 in the resulting pellets and supernatants was analyzed by gel electrophoresis and immunoblotting. p60 bound readily to the liposomes in the low-salt Mops/$MgCl_2$/EGTA buffer (Fig. 2, lanes 1 and 2). In contrast, binding was completely abolished at 300 mM NaCl (lanes 3 and 4), suggesting that the binding of p60 to liposomes is mediated by both hydrophobic and ionic interactions. The inclusion of 1 mM ethanolamine in the binding buffer interfered slightly with p60 binding (lanes 5 and 6). No p60 was recovered in the pellet when a control binding reaction was performed in the presence of 0.1% Triton X-100 (lanes 7 and 8), which dissolves the liposomes. Binding of p60 to the liposomes was completely prevented by 1 mM chlorpromazine (lanes 9 and 10), although this drug exerted no effect on the binding of p60 to microtubules (see Fig. 1*b*, lanes 11 and 12). A control reaction in which no p60 was added to the liposomes demonstrated that asolectin liposomes *per se* do not contain any components that react with the p60 antibody (Fig. 2, lanes 11 and 12) or that can be detected by staining with Coomassie blue (data not shown). Thus, the above experiments established that p60 can readily bind to asolectin liposomes. However, this interaction is not indiscriminate but is strongly dependent on liposome composition. This is illustrated by the observation that p60 failed to bind to liposomes reconstituted either from phosphatidylcholine alone or from mixtures of phosphatidylcholine and phosphatidylethanolamine. With both types of liposomes, no binding of p60 was observed under conditions of low ionic strength (Fig. 3, lanes 3 and 4 and lanes 9 and 10). At higher ionic strength (300 mM NaCl), a weak binding of p60 to phosphatidylcholine liposomes, but not to phosphatidylethanolamine liposomes, was observed. This contrasts to the situation seen with asolectin liposomes, where the binding of p60 is completely abolished in 300 mM NaCl. The binding of p60 to phosphatidylcholine liposomes thus most likely reflects nonspecific hydrophobic interactions between p60 and liposomes, which are enhanced by the increased ionic strength. The detailed lipid requirements for p60 binding still remain to be explored.

p60 Can Crosslink Microtubules and Membrane Vesicles. The above results established that p60 can interact separately with microtubules as well as with liposomes. This suggested that p60 may mediate crosslinking between these two structures. Electron microscopic inspection of the trypanosomal microtubule preparations used in the above binding experiments showed that they always contained numerous membrane vesicles, as has been reported for similar microtubule preparations from other cell types (25, 26). Fig. 4*a* presents such a microtubule preparation from trypanosomes, which contained individual microtubules (200–400 nm long) as well as membrane vesicles, all well-dispersed. As increasing amounts of p60 were added to such preparations, fewer free microtubules and vesicles were detected; most aggregated into large, impenetrable tangles. An example of one of the less complex structures (Fig. 4*b*) illustrates the extensive crosslinking of membrane vesicles and microtubules that is induced by the added p60 protein. The many complex tangles that were observed prevented a more quantitative analysis of the stoichiometry of the reaction. No microtubule-bundling activity of p60 similar to that reported

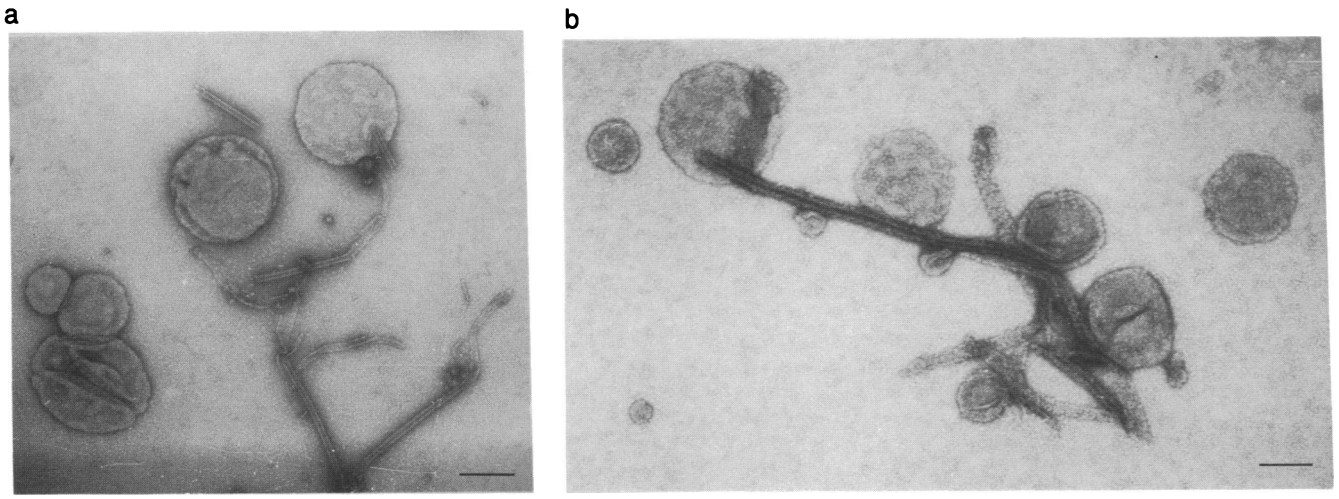

FIG. 4. p60 can crosslink microtubules and membrane vesicles. (*a*) Negatively stained microtubules. (*b*) Negatively stained microtubules after a 10-min incubation with p60. (Bar = 0.1 μm.)

for some other microtubule-associated proteins (28–30) was detected under these conditions. However, the structure presented in Fig. 4*b* demonstrates that this protein can interact simultaneously both with microtubules and with membranes, thus serving as a crosslink between the two.

DISCUSSION

The results show that p60, a protein that has been identified as a component of the microtubular membrane skeleton in *T. brucei*, is capable of binding *in vitro* both to microtubules and to liposomes. The binding to microtubules exhibits a similar dependence on ionic strength as is observed for the endogenous p60 in the trypanosomal cytoskeleton. The binding to liposomes is dependent both on their lipid composition and on the ionic strength, indicating that both hydrophobic and ionic interactions are involved in the binding of p60 to liposomes. Perhaps most significant, p60 can crosslink membrane vesicles to microtubules. In conjunction with its intracellular location in the microtubular membrane skeleton, these results suggest that p60 may constitute a component of the structure that links the skeletal microtubules to the overlying cell membrane.

Though p60 may be only one of several components of such a link, it is interesting that its occurrence is confined to the family of the Trypanosomatidae—i.e., to protozoans that all exhibit a similar microtubular membrane skeleton (23). A protein that may be a homologue of the trypanosomal p60 described in this study was detected in the membrane skeleton of the related trypanosomatid *Crithidia fasciculata* (24). The narrowly defined systematic distribution of p60 and its homologues may suggest that the family of the Trypanosomatidae has developed its own type of microtubule/membrane contacts. A more detailed analysis of these structures is expected to further our understanding of microtubule/membrane interactions in general and, considering that trypanosomes are important parasites, may provide new leads for a chemotherapeutic attack against these organisms.

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