Disassembly and Reconstitution of a Membrane-Microtubule Complex

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ABSTRACT The cell membrane of the unicellular algae Distigma proteus is associated with arrays of parallel microtubules. Fragments of the membrane-microtubule complex have been isolated and partially purified. The microtubules were stable in vitro at room temperature as well as at 0°C, but were specifically and rapidly disassembled by Ca²⁺. After removal of all endogenous microtubules, the membrane-microtubule complex could be reassembled from brain microtubule protein and denuded Distigma membrane fragments. The readded microtubules bound in a fixed orientation, and only to those regions of membrane that are normally associated with microtubules in vivo.

An association of microtubules with membranes has been inferred from electron microscopic examination of thin sections of a variety of cells (1–6) and of negatively stained membranes from flagella (7). Biochemical evidence for association of tubulin with membranes has also been reported from a different group of cells (8–11). Membrane-associated microtubules are common among protozoa, and are a particularly prominent feature of the Euglenophyceae, unicellular flagellated algae (12–15). One member of this class, Distigma proteus, offers several advantages for study of membrane-microtubule interactions. Prominent arrays of microtubules lie immediately beneath the cell membrane, and probably participate in the striking shape changes (“euglenoid movements”) which these algae undergo (15, 16). The membrane complex is uniquely well suited to structural analysis, and can be easily isolated for biochemical manipulations. The three-dimensional structure of the membrane-microtubule complex from this organism has been previously described (17). I report here some results from a biochemical investigation of the complex, including its partial disassembly and reconstitution.

MATERIALS AND METHODS

Cell Culture and Membrane Preparation: Distigma proteus (strain No. UTEX 508) was obtained from the Culture Collection of Algae at the University of Texas at Austin. Cultures were maintained on soil water medium at room temperature as described by Pringsheim (18).

Centrifugation in a 1.5-ml swing-out tube at 600 g for 2 min was used to harvest cells, which were then washed three times with distilled water. The cells were resuspended in water and allowed to stand undisturbed for several hours, during which time nonswimming cells and debris settled to the bottom. The supernatant, in which 100% of the cells were actively swimming, was carefully removed and the cells collected by centrifugation. The pellet was resuspended in 0.1 M NaMES, pH 6.4 (2-(N-morpholino)ethanesulfonic acid; pH adjusted with NaOH) 1 mM Na₂EGTA, 5 mM MgCl₂, 1 mM TAME, 0.2 mM PMSF, 1.5 μM pepstatin A, 4 μM leupeptin, pH 6.4 at 20°C (buffer A) and washed once with this buffer. Flagella were removed by passing the cell suspension rapidly through a 26-gauge needle. Centrifugation at 500 g for 1.5 min sedimented the cells (no longer able to swim) leaving the flagella in the supernatant solution.

The pellet was washed twice in the same buffer to completely rid it of flagellar remnants, and the cells were then broken by shaking with glass beads at ~30 Hz for 4 min. Membrane fragments were collected by centrifugation at 4,000 g for 5 min and washed once in the same buffer. After this wash the pellet was resuspended and used immediately for assembly and disassembly experiments. Further purification was carried out for analysis of the membrane complex by SDS gel electrophoresis. The material was centrifuged on a linear density gradient of metrizamide (5-ml gradient, density range 1.131 to 1.269 g/ml) at 35,000 rpm in a Beckman SW 50.1 rotor (Beckman Instruments, Palo Alto, CA) for 1 h. The lower third of the gradient, excluding the pellet, was removed and the membrane fragments washed in buffer A.

This fraction was recentrifuged on a shallower gradient (density range 1.159 to 1.228). The membrane fragments accumulated as fluffy clumps in the lower 10% of this gradient after centrifugation at 35,000 rpm for 1 h.

Membrane Complex Disassembly and Reconstitution: Microtubules were partially or completely removed from membrane complex fragments, previously washed in buffer A, by washing a second time and resuspending with buffers of the desired final [Ca²⁺]. The various [Ca²⁺] were achieved by adding appropriate amounts of CaCl₂ to the usual buffer A, calculated from a value of 3.3 x 10⁻⁶ for the CaEGTA dissociation constant at pH 6.4 (19). After resuspension, the tube was incubated with gentle mixing for 10 min and then centrifuged for 4 min at 4,000 g to collect the membrane fragments for examination or subsequent use.

Fragments to be used for reassembly experiments were freed of microtubules by a 10-min incubation in buffer A containing 5 mM free Ca²⁺. They were then washed once with calcium free buffer A, and once with 0.1 M NaPIPES, 1 mM MgCl₂, 2 mM EGTA, 1.0 mM GTP pH 6.62. Bovine brain tubulin was added to this suspension of membrane fragments to a final tubulin concentration that had been previously determined to be the minimum required for polymerization in the absence of membranes (i.e., the final tubulin concentration was slightly higher than the "critical concentration" of tubulin under these conditions).
Chemical Co. (St. Louis, MO). The procedure of Burk et al. was carried out using the buffersystem of Laemmli (27). Silver staining followed.

The lipid bilayer. The structure of the membrane and associated proteins can be isolated from the membrane-microtubule complex. Immediately after breaking the cell, most of the microtubules are attached to the membrane, and remain associated with it through repeated washes with buffers of approximately physiological pH and ionic strength. At the expense of some loss of microtubules, the membrane strips can be separated from other cell debris by density gradient centrifugation in metrizamide. The composition of such a preparation is shown by electron microscopy of a pellet of the material in Fig. 2a and by SDS gel electrophoresis in Fig. 3a. Although some cytoplasmic remnants are seen in Fig. 2a, the greater part of the material is the membrane-microtubule complex. In Fig. 3, there is a prominent double band of the mobility expected for tubulin, apparent molecular weight 54,000. Examination of isolated membrane fragments by electron microscopy confirms the presence of many microtubules at the same locations as those observed in vivo (Fig. 2b).

Disassembly of the Membrane-Microtubule Complex

Some of the microtubules present in vivo are lost during cell breakage or membrane purification and there seems to be a continued slower loss thereafter. On average, each fold of membrane retains five microtubules after isolation, compared with eight in vivo. The composition of the buffer in which the membrane fragments are isolated was chosen by selecting conditions that protected against the loss of attached microtubules. In this buffer, most of the microtubules that are present immediately after isolation persist for >24 h either at room temperature or at 0°C.

In addition to their enhanced cold-stability, the microtubules are also relatively resistant to drugs. Many agents that depolymerize microtubules in other organisms have little effect on these membrane-associated microtubules from Distigma. Treatment of isolated membrane fragments with colchicine (125 μM) p-fluorophenylalanine (1.3 mM), griseofulvin (280 μM), nocodazole (41 μM), or benomyl (230 μM) did not change the average number of microtubules present (data not shown). However, the microtubules were completely removed within minutes by washing with buffer containing 1 mM Ca²⁺ (Fig. 4). Mg²⁺ at 5 mM does not substitute for Ca²⁺ in this role. Lower concentrations of Ca²⁺ removed fewer microtubules under these conditions. A quantitative comparison of the effectiveness of Ca²⁺ at different concentrations is shown in Fig. 5. A small effect is noticeable as low as 1 × 10⁻⁶ M under the conditions of this experiment.

The material extracted from membrane fragments by Ca²⁺-containing buffer was examined to determine the fate of the removed microtubules. Membrane fragments containing a normal complement of microtubules were washed in a small volume of Ca²⁺-containing buffer, and the supernatant solution was examined by electron microscopy. No structures resembling microtubules could be found after applying samples of supernatant solution to hydrophilic carbon-coated grids and negative staining. SDS gel analysis of this supernatant solution revealed a large amount of protein in two closely spaced bands of apparent molecular weight 54,000 (Fig. 3c). Thus the microtubules removed from the membrane by Ca²⁺ appear to be disassembled into soluble tubulin. Very little protein is extracted from the complex by washing with buffer of low Ca²⁺ (Fig. 3d).
FIGURE 1  (a) Scanning electron micrograph of Distigma proteus. The cell shape changes constantly ("euglenoid movement"). The shapes observed here are a random sample trapped by rapidly freezing the cells. Folds in the cell surface are visible as helical striations, which change in pitch during the shape changes. (b) Cross-section of Distigma proteus showing the characteristic serrated cell boundary resulting from the 18 longitudinal folds in the surface. (c) higher magnification view of a thin section from a tannic acid-treated cell, to show details of the surface folds and underlying microtubules. A hooked microtubule is normally found at the position indicated by the arrow. Bars, 50 nm. x 2,350 (a); x 16,500 (b); x 194,000 (c).

When Ca²⁺ washed membrane fragments were examined by thin sectioning, small wisps of material were observed projecting from their cytoplasmic surface, predominantly in the areas where microtubules were previously located (Fig. 4). These projections were not seen when microtubules were present; they may be the binding sites for microtubules, exposed by removal of the tubulin. Their location and approximately regular spacing are suggestive, but at present we have no more certain way of establishing their identity. The previously reported crystalline array of membrane proteins present on the membrane complex is not altered by washing with Ca²⁺ (17).

Reassembly of Microtubules on the Membrane Complex

The availability of membrane complex completely free of microtubules made it possible to look for microtubule binding sites in a straightforward way. Membrane fragments freed of microtubules by washing with Ca²⁺-containing buffer were then washed in EGTA-containing buffer and mixed with bovine brain tubulin at 0°C. When this mixture was warmed to 32°C, the tubulin polymerized and formed arrays of microtubules attached to the membrane. The resulting complex appeared similar to the original structure before washing with Ca²⁺ (Fig. 6), though sometimes the number of attached microtubules was greater than in membrane fragments that were isolated, as described in Materials and Methods. The readded microtubules were found in approximately the same area as the original large cluster of microtubules, under one side of the membrane fold. The newly added microtubules remained associated with the membrane in this way when the complex was washed with Ca²⁺-free buffer at 32°C. It appears that the membrane has specific microtubule binding sites that remain in place after the Ca²⁺ wash that removed the original microtubules. In agreement with the previous structural anal-
FIGURE 3  SDS gel analysis of the isolated membrane microtubule complex from *Distigma proteus*. Apparent molecular weight (kilo-daltons) is indicated on the left of the figure. The extreme high and low molecular weight regions of this gel are not shown. (a) Intact membrane microtubule complex. Tubulin runs as a pair of bands of equal intensity (indicated by the two arrowheads) separated by an unidentified sharp band of higher intensity. (b) Membrane complex after washing with buffer containing Ca²⁺. The tubulin doublet is relatively depleted, but the central band that it brackets is unchanged. A small amount of tubulin remains, primarily due to contamination of the membrane preparation with flagella, which do not dissociate in Ca²⁺. (c) The peptides extracted from membrane-microtubule complex by washing with Ca²⁺. The tubulin doublet is much clearer than in a since the intervening peptide remained on the membrane. (d) Material removed from the membrane by washing with buffer containing 1 mM EGTA.

ysis (17), *Distigma* membrane-bound microtubules seem to be similar to microtubules from higher eucaryotes, since the bovine brain material is readily substituted for them.

In considering ways in which the membrane-microtubule complex might be assembled in vivo, as well as in thinking about its possible functions, it is useful to know the polarity of the microtubules. Heidemann and McIntosh (20) have described a set of polymerization conditions that produce microtubules with an asymmetric hook-shaped appendage whose appearance in thin sections was shown to provide a reliable indicator of polarity. This technique was employed to determine the polarity of microtubules reassembled on *Dis-

FIGURE 2  Isolated cell membranes from *Distigma proteus*. (a) Thin section of a pellet of the membrane preparation. The membrane fragments are elongated strips including several surface folds each, which frequently roll up into an irregular loose spiral. Remnants of cell cytoplasm are sometimes entrapped by this curling up. (b) Higher magnification view of an isolated fragment of membrane to which microtubules remain attached. Bar, 1 µm; × 12,000 (a). Bar, 100 nm; × 95,000 (b).
FIGURE 4 (a) Thin section of fragments of membrane after washing with Ca²⁺-containing buffer. Virtually all microtubules have been removed. (b) At higher magnification, faint wisps of material (arrows) projecting from the membrane can now be seen in the area formerly occupied by microtubules. Bar, 500 nm; x 24,000 (a). Bar, 100 nm; x 89,000 (b).

FIGURE 5 Effect of various Ca²⁺ on membrane-bound microtubules of isolated membrane fragments. Membrane-microtubule complexes prepared as described in Materials and Methods were washed with buffers containing different levels of free calcium as indicated on the abscissa. Washed samples were fixed, embedded, sectioned, and the average number of microtubules per membrane fold determined by counting in micrographs all appropriately oriented specimens. The average and its standard error are expressed in the figure as a percentage of the microtubules present in an EGTA-washed sample of the same preparation. Approximately one-hundred folds were counted for each point.

**DISCUSSION**

The microtubules of *Distigma proteus* were relatively resistant to disassembly by drugs or by low temperature. There was a slow loss of microtubules from isolated membrane-microtubule complexes over a period of several hours after isolation, which was not affected by keeping the preparation at different temperatures. This enhanced stability compared with cytoplasmic microtubules of higher eucaryocytes may reflect enzymatic alteration of some component of the complex in a...
Reassembly of bovine microtubule protein into membrane-microtubule complexes. Isolated membrane fragments, washed free of endogenous microtubules as in Fig. 4, were incubated with bovine brain tubulin under polymerizing conditions. Many microtubules form on the membranes in a pattern similar to the original arrangement. Bar 100 nm. × 56,000.

The physiological significance of a depolymerization induced by 10⁻³ M Ca²⁺ is unclear. It seems unlikely that the intracellular Ca²⁺ level could ever be so high (16), and there is no evidence to indicate Distigma microtubules are ever depolymerized. It has been shown previously that shape changes of the cell are regulated by fluctuation in Ca²⁺ over the range 10⁻⁷ to 10⁻⁴ M (16), much lower than that necessary for this rapid depolymerization observed in vitro. The relationship between the effect of Ca²⁺ on membrane movement and on microtubule stability is presently obscure, but potentially interesting with respect to the mechanism of euglenoid movement.

In addition to tubulin, several other peptides are released from the membrane complex by Ca²⁺ in significant amounts (Fig. 3 c). One class of proteins that might be expected to appear in the supernatant solution with tubulin is the group that normally binds to the intact microtubules, to anchor them to the membrane, or to produce motion in a dynein-like manner, or for other unknown functions. Antibodies are presently being raised against these peptides, visible in Fig. 3 c, as a means of locating them in the intact complex.

Previously reported structural studies of the complete membrane-microtubule complex (17) described a crystalline array of integral membrane proteins that appear to serve as scaffolding for the attachment of microtubules via an intermediate linking protein. This array of integral membrane proteins was not disturbed by washing with Ca²⁺. The most obvious interpretation of the reconstitution experiments after removal of endogenous microtubules is that the linking proteins also remain in place after washing with Ca²⁺. However, this may not be correct, since the preparation of brain microtubule protein used for reassembly is known to contain ~20% high molecular weight microtubule-associated protein (22). Among the several peptides included in the microtubule-associated protein fraction may be one or more capable of replacing any Distigma microtubule-binding proteins extracted by the Ca²⁺ wash. It should be possible to check this directly by attempting reassembly using pure tubulin free of microtubule-associated proteins.

One striking feature of the reconstituted membrane-microtubule complex is that the newly added microtubules were restricted to the regions under the membrane normally occupied by microtubules in vivo, the left side of each fold in the orientation of Fig. 6. When higher tubulin concentrations were employed some microtubules were found under the right side of the membrane folds, but one also finds a large number of free, obviously unbound, microtubules, suggesting that some of those now under the membrane may be the result of coincidental approximation rather than true binding. In either case, it is clear that the two sides of the membrane are quite
different in some characteristic important for microtubule binding. The structural basis for this difference is not apparent, unless the poorly defined wisps seen in Fig. 4 somehow determine microtubule-binding sites. The previous work with negatively stained membrane fragments did not detect any difference between membrane overlying microtubules and that in microtubule free regions (17). A major goal of future experiments is to discover the basis of the microtubule localization.

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