Detection of Single Microtubules in Living Cells: Particle Transport Can Occur in Both Directions along the Same Microtubule

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ABSTRACT Video-enhanced contrast/differential interference-contrast microscopy was used in conjunction with whole mount electron microscopy to study particle transport along linear elements in fibroblasts. Keratocytes from the corneal stroma of *Rana pipiens* were grown on gold indicator grids and examined with video microscopy. Video records were taken of the linear elements and associated particle transport until lysis and/or fixation of the cells was completed. The preparations were then processed for whole mount electron microscopy.

By combining these two methods, we demonstrated that linear elements detected in the living cell could be identified as single microtubules, and that filaments as small as 10 nm could be detected in lysed and fixed cells. The visibility of different cytoplasmic structures changed after lysis with many more cellular components becoming visible. Microtubules became more difficult to detect after lysis while bundles of microfilaments became more prominent. All particle translocations were observed to take place along linear elements composed of one or more microtubules. Furthermore, particles were observed to translocate in one or both directions on the same microtubule.

Cytoplasmic transport is a process of ubiquitous occurrence in eucaryotic cells and of fundamental importance to cell function. One manifestation of cytoplasmic transport, the translocation of optically detectable organelles and vesicles (collectively called particles), has been extensively studied (20, 27). Microtubules have long been believed to play an important role in particle transport in such diverse cells as chromatophores (4, 22), neurons (15), tissue culture cells (13), and in numerous protists, e.g., the foraminefer, *Allogromia* (34, 35). These cells possess impressive arrays of cytoplasmic microtubules, the presence and orientation of which have been shown by electron (4, 5, 13, 34, 36) and immunofluorescence (9) microscopy to be consistent with the hypothesis that particle motion occurs along them. Furthermore, microtubule depolymerizing drugs have been shown to inhibit particle motion in all these cells (13, 17, 25, 33).

Until recently, the major limitation of particle transport studies had been the inability to detect and identify cytoplasmic microtubules in the living cell. Video-enhanced microscopy has now made it possible to observe microtubules in vitro in a slurry (1) or in a living cell (18). In a previous study, using Allen video-enhanced contrast/differential interference-contrast (AVEC-DIC) microscopy in conjunction with immunofluorescence microscopy, we demonstrated directly that particles move in keratocytes along linear elements composed of microtubules (18). The particles displayed a number of interesting behaviors including motion in either direction along the same microtubular linear element. However, this study was limited in that the number of microtubules or other cytoskeletal elements making up a linear element could not be determined. Thus, it was possible that a particle could move in one direction along one microtubule in a linear element composed of microtubules and on another microtubule in the reverse direction.

In the present study, we observed particles moving along linear elements in keratocytes preceding fixation and preparation for whole mount electron microscopy. We show that by comparing the video record to the corresponding whole mount electron micrographs, one can determine the numbers and kinds of cytoskeletal elements that make up a light
were stripped away. The remaining stroma that contained the keratoeytes was placed between two number 0 coverslips in petri dishes and grown at 24°C in antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml Fungizone). All media components were supplied by Gibco Laboratories, Grand Island, NY. Gold London Finder grids (Ladd Research Industries, Inc., Burlington, VT) were Formvar- and carbon-coated, and sterilized by exposure to ultraviolet light. The keratoeytes were collected from the coverslips with 0.25% trypsin made up in Ca++ and Mg++ free Earle’s balanced salt solution (Gibco Laboratories) and seeded onto the prepared electron micro-paraffin (1:1:1). The preparations were examined with AVEC-DIC (1, 2) scope grids.

In preparation for light microscopy, a coverslip was fastened to a slide holder with vinyl-lanolin-paraffin (1:1:1), and the grids with attached cells were placed in a drop of medium on the coverslip. A chamber was formed by laying a clean coverslip on top of strips of Scotch tape that ran the length of two sides of the bottom coverslip. These two sides were sealed with vinyl-lanolin-paraffin (1:1:1). The preparations were examined using AVEC-DIC (1, 2) microscopy using an inverted Zeiss Axiosmat (Carl Zeiss, Inc., Oberkochen, Wurttenberg, West Germany) equipped with a 100 ×/1.3 NA planapochromatic objective. The bias retardation was set to ≥1/3. The image was projected either onto a C-1000-01 binary computer-compatible chaitemic video camera driven by a model 1440 Poly!aroeessor Frame Memory (Hamamatsu Systems Inc., Waltham, MA), or onto a Chaimecn video camera driven by a newer device, the C1966 AVEC/VIM Photonic Microscope System Image Processor (Photonic Microscopy, Inc., Chicago, IL).

Once the video records of the cells were taken, the preparations were processed for whole mount electron microscopy. The cells were either lysed and then fixed, lysed and fixed simultaneously, or fixed without lysis. Lysis and/or fixation medium was added to one open side of the coverslip preparation and drawn through by placing filter paper strips at the other open side. The and/or fixation medium was added to one open side of the coverslip preparation and drawn through by placing filter paper strips at the other open side. The microtubule stabilizing buffer used for lysis and/or fixation consisted of 25 mM PIPES (pH 6.9), 1.0 mM EGTA, 1% polyethylene glycol 6000, and 0.5 mM MgCl. This buffer contained 0.5% Triton X-100 and 1.0% glutaraldehyde for lysis and fixation, respectively. Video records were taken of lysis and fixation. After lysis the preparations were removed from the light microscope, washed with microtubule stabilizing buffer, and postfixed for 1 min with 1.0% osmium in 100 mM Cacodylate buffer (pH 6.0, 4°C). The preparations were then washed with distilled water, dehydrated through a graded ethyl alcohol series, and critical point dried. At the 70% alcohol dehydrtation stage, the preparations were stained for 1 min with 0.5% uranyl acetate in 70% alcohol.

The cells were examined with a JEOL 100 CX electron microscope operating at 100 kV, and the whole mount electron micrographs corresponding to the video records were obtained.

RESULTS

All structures visible in the light micrographs of living cells were referred to in general terms as either linear elements or particles. After lyis and/or fixation, the linear elements were subsequentlv identified from the corresponding whole mount electron micrographs on the basis of size and organization. According to convention, the 6-nm filaments were referred to as microfilaments (MFs), and the 10-nm filaments as intermediate filaments (IFs). The microtubules (MTs) were identified both on the basis of their size (15–20 nm) and their characteristic pattern in the cell. (See figure legends for a more detailed description of the results.)

In the flattened peripheral regions of these cells, various linear elements could be seen (Fig. 1 a). In preparation for whole mount electron microscopy, the cells were lysed and fixed while they remained in focus in the light microscope. The living cell in Fig. 1 a is shown again in Fig. 1 b after lysis and fixation in a microtubule stabilizing solution that contained 0.5% Triton X-100 and 1.0% glutaraldehyde. The replacement of the soluble components of the cell with the lysis medium led to an increase in contrast for all linear elements visible in the living cell, and caused many other cellular elements to become visible for the first time. In fact, one linear element became evident only after lysis. One result of this increase in contrast was that some of the linear elements pointed out in the living cell (Fig. 1 a) were more difficult to detect. This is because the increased number of visible cellular components after lysis tends to obscure, partially or totally, some linear elements.

Whole mount electron microscopy was used to identify the linear elements seen in the living and lysed cells. Fig. 2 a is a low magnification electron micrograph of the same region shown in the light microscope in Fig. 1. The linear element that became apparent only upon lysis was an intermediate filament (Fig. 2, a and c), and two linear elements that became more evident upon lysis corresponded to bundles of microfilaments (Fig. 2 a). One linear element, visible in the living cell but totally obscured in the lysed cell, was composed of two microtubules (Fig. 2 a). However, the two curvilinear elements pointed out in the live and lysed cell (Fig. 1, a and b) are single microtubules (Fig. 2, a and b). Thus, single microtubules are detected in the living cell by AVEC-DIC microscopy, while a 10-nm filament could be detected only in the lysed cell.

We observed particles translocating along linear elements composed of microtubules in living cells. In Fig. 3, a and b, a particle is shown moving down a prominent linear element. This particular cell was then lysed for 30 s with 0.5% Triton X-100 in a microtubule stabilizing solution before fixation with 1.0% glutaraldehyde. Once again, upon lysis many more cellular elements became apparent, thus obscuring the linear element the particle moved along (Fig. 3 c). Also, some of the less evident linear elements in the living cell became the most visually prominent elements in the lysed cell. The corresponding whole mount electron micrograph demonstrates these latter elements to be bundles of microfilaments (Fig. 3, d and e), while the linear element along which particles translocated in the living cell is composed of two microtubules (Fig. 3, d and e).

Particles were also observed translocating along linear elements composed of single microtubules. In Fig. 4, a and b, a particle is shown moving along one of three parallel linear elements. A total of four particles translocated in the same direction along the lower two linear elements. These linear elements were identified as single microtubules in the corresponding whole mount electron micrographs (Fig. 4, d and e). In this preparation, the cell was fixed without lysis in a microtubule stabilizing solution that contained 1.0% glutaraldehyde. In this case, the microtubules only became obscured by other cellular components in a region of vesiculation; otherwise, the microtubules appeared to retain their relationships to the rest of the cell to the extent that they remained the most prominent linear elements in the region.

Finally, in another preparation, a particle was observed to translocate in both directions along a linear element (Fig. 5 a–e). This preparation was lysed and fixed in a microtubule stabilizing buffer that contained 0.5% Triton X-100 and 1.0% glutaraldehyde. The corresponding whole mount electron micrograph shows that this linear element consisted of a single microtubule (Fig. 6). This was one of two preparations in...
which transport in both directions along a single microtubule was documented. Thus, particles can not only translocate along single microtubules, but they can also do so in either direction.

DISCUSSION

Particle Transport

We have used video microscopy in conjunction with whole mount electron microscopy to demonstrate that linear elements detected in the living cell can subsequently be identified as single microtubules. Furthermore we detected and identified filaments as small as 10 nm in diameter in lysed cells. The main significance of these observations is that some of the functions of cytoplasmic microtubules can now be studied directly. Using this approach to study the role of microtubules in particle transport, we have demonstrated that particles can move in both directions on the same microtubule.

In a previous study with keratocytes (18), we showed using AVEC-DIC microscopy in conjunction with immunofluorescence microscopy, that particles are transported exclusively along linear elements composed of microtubules. Particles were also observed to undergo a number of interesting behaviors including motion in either direction on the same microtubular linear element. However, it was not possible to determine the number of microtubules making up a linear element.

Particle motion in both directions along the same microtubular linear element is of interest when one considers the possibility that (a) the structural polarity of microtubules is translated into a functional polarity, and (b) dynein, an ATPase mechanoenzyme, (14) is the mechanochemical transducer responsible for particle translocation.

Does the structural polarity of microtubules manifest itself as a functional polarity? Some models for mitosis assume that the direction of polarity of the microtubules plays an important part in chromosome motion (23, 24). Our work showing particle motion in both directions along the same microtubule indicates, at least for particle transport in keratocytes, that the structural polarity of the microtubules does not dictate the direction of motion. Also, recent studies with nerve axons (7, 11, 19), melanophores of the angel fish (10), and axopodia of Echinosphaerium (10) have shown most of the microtubules to be of the same polarity though bidirectional particle motion occurs in these cells.

Whether dynein could be the mechanochemical transducer responsible for particle translocation also hinges on questions of polarity. Dynein is an asymmetric molecule that has been used to determine the structural polarity of microtubules (16). Ciliary dynein is attached to the A-subfiber by its A-end (32), and it interacts with the B-subfiber via its B-end which contains the ATPase activity. In a simple microtubule-dynein-particle hypothesis, the dynein could presumably be attached to either the microtubule or particle by its A-end. Under the proper conditions the dynein would then be able to generate...
movement by interacting with either the particle or microtubule via its B-end.

The evidence suggesting a possible dynein involvement in particle transport includes work showing cross-bridges between synaptic vesicles and microtubules (21), and between mitochondria and microtubules (26, 29, 30). The nature of this cross-bridging material has not been determined, nor has any ATPase activity been associated with it. Experiments with permeabilized fibroblasts (12), neuroblastoma cells (31), and melanophores (8) have shown that vanadate inhibits particle motion at concentrations that also inhibit dynein ATPase activity. However, it also has been shown that vanadate does not inhibit particle motion at concentrations that inhibit ciliary beating in the same oviduct cell (6). Erythro-9-[3-(2-hydroxymomyl)]adenine, another dynein ATPase inhibitor, inhibits particle motion in intact erythroophores (3) and melanophores (8).

Our experiments raise the question of whether dynein is
FIGURE 3 Light micrographs and whole mount electron micrographs demonstrating particle motion along a linear element composed of microtubules. A particle (large arrows) is moving down a prominent linear element (small arrows) in the live cell in a and b. The lysed and fixed cell is shown in c. Here again, many cellular components became visible upon lysis; this tends to obscure the linear element the particles moved upon. A portion of this linear element is still visible (small arrow), while some linear elements (arrowheads in b and c) became much more prominent after lysis. The corresponding electron micrograph of this cell region is shown in d. The linear element that the particle moved along is composed of two microtubules (large and small arrows, MTs). A higher magnification micrograph of the region of these two microtubules defined by the small arrows in d is shown in e. The microtubules in this region are still detectable in the light microscope after lysis (c) because the area immediately surrounding them contains very little material. The linear elements that became more prominent after lysis are composed of microfilaments (d and f, large and small arrowheads, MFs). A region of one of these bundles of filaments defined by the small arrowheads in d is shown in f. Bars: (c and d) 2.0 μm; (e and f) 0.2 μm. (c) × 4,300; (d) × 9,300; (e and f) × 60,000.
FIGURE 5 Light micrographs of a cell in which motion of a particle (white arrowheads, P) in either direction along the same linear element (white arrows) was observed. The particle is moving down the linear element (a), pausing (b), and returning up the same linear element (c–e). The cell is shown in e just before lysis and fixation. The particle is seen just before reaching the cell mass (black arrowhead) present in both light and electron micrographs. The black arrow at the bottom points out a filopodium that is also present in both light and electron micrographs. The cell is shown after lysis and fixation in f. The linear element that the particle moved along (as well as the other linear elements) became much harder to detect after lysis. A section of the linear element that the particle moved along is still visible at the arrow. Bar, 2.0 μm. × 12,000.

FIGURE 4 Light and whole mount electron micrographs demonstrating particle motion along a single microtubule. (a and b) A particle (arrows) is shown moving along the lowermost of the three linear elements labeled by the black arrowheads. The same region is shown in c after fixation without lysis. The particle shown in (a and b) had moved out of the field of view just before fixation. Note that there was some vesiculation at the lower left (white arrowheads). The letter B (c and d) indicates holes in the plastic that are useful as landmarks when comparing the light micrographs to the whole mount electron micrographs. The whole mount electron micrograph corresponding to the light micrographs (a–c) is shown in d. Note that the three linear elements seen in the live and fixed cell are single microtubules (black arrowheads). A higher magnification of the region of the lowermost microtubule defined by the small arrowheads is shown in e. Other non-microtubular elements closely surround it and may run parallel to it for a short distance. Some arrangements of cytoskeletal components may have the same width as a microtubule, and so they appear to be microtubules at high magnification. In e, ~1 cm to the left of the second arrowhead, the microtubule appears to split in two and continue to the left as two microtubules. By using the lower magnification micrograph (d) to follow the course of the microtubule, one sees that there is only one microtubule present and that another cytoskeletal component, only a few tenths of a micrometer in length, happens to intersect the microtubule just to the left of the second arrowhead (e). This demonstrates the importance of using both the microtubule width and pattern in the cell to identify microtubules. In the living cell, two particles moved along the bottommost microtubule and two particles moved along the microtubule immediately above it. In this preparation, the microtubules remained as very prominent elements after fixation without lysis. They only became obscured in the region of vesiculation where an increase in contrast caused other cellular components to become visible.

Bars: (c and d) 2.0 μm; (e) 0.2 μm. (c) × 3,800; (d) × 20,000; (e) × 90,000.
Figure 6 (a) Whole mount electron micrograph, corresponding to the cell region in Fig. 5, demonstrates that the linear element that the particle moved along in either direction is a single microtubule (white arrows). The letters a–e correspond to the sequence of particle positions seen in the living cell with the white arrowheads showing the direction of motion. The particle reversed directions at b and was observed at e just before reaching the cell mass shown here by the star (corresponding to the area shown by the black arrowhead in Fig. 5e). The filopodium noted in Fig. 5e is shown at the lower right (black arrow). The region of the cell in which the particle was last observed (Fig. 5e) is shown at higher magnification in b. The star labels the cell mass seen in Fig. 5 and Fig. 6a. The arrows point out the microtubule that the particle moved along. Note that other cytoskeletal components closely surround the microtubule. Bars: (a) 1.0 μm; (b) 0.1 μm. (a) × 22,000; (b) × 100,000.

still a candidate for the mechanochemical transducer. If the structural polarity of the dynein-microtubule interaction also dictates a functional polarity, then our studies would appear to rule out a dynein involvement in particle transport. However, there is some confusion on the point of dynein-microtubule polarity. In one study with microtubules assembled in vitro (16), dynein was shown to cross-bridge microtubules only when they were of the same polarity; in another study dynein was shown to cross-bridge ciliary microtubule doublets of the same and opposite polarity (37). The doublets were still capable of sliding relative to one another upon addition of ATP. Resolution of this dynein-microtubule polarity question...
is necessary to evaluate the significance of particle transport in both directions along the same microtubule.

It is possible that a microtubule-dynein interaction may not be involved in particle transport. It may be that microtubules only serve to define the paths particles may follow through the cytoplasmic matrix and that some other mechanochemical transducer is responsible for particle transport. We can only say from our light microscopy studies that particles always travel in optical contact with microtubules (i.e., within 200 nm). However, the corresponding electron micrographs show that the microtubules are surrounded by as yet unidentified elements which are much closer than 200 nm to the microtubules (Figs. 4 e and 6). It is possible that these elements are also involved in particle transport.

Clues to the identity of any mechanoenzyme that may be involved in particle transport remain elusive. We plan to approach this problem by preserving the particles so that the particle-microtubule interactions can be determined. We hope that by correlating known particle behaviors at the time of fixation to the corresponding whole mount electron micrographs, we will gain further insight into the problem of particle transport.

Cytoplasmic Matrix

Some interesting observations of the present study came from comparing the living and lysed cells to one another and to the whole mount electron micrographs. Although the microtubules were the most prominent linear elements in the living cell, linear elements composed of microfilaments were more prominent in the lysed cell. All the linear elements gained in contrast because the lysis removed the Triton X-soluble components of the cell, thereby increasing the refractive index gradient between linear elements and background. This increase in contrast caused many cellular elements to become evident for the first time, thus obscuring the microtubular linear elements. However, this does not explain why bundles of microfilaments, which were less obvious in the living micrographs, we predicted that the bundles of microfilaments would be more prominent than the microtubules in the DIC image, as indeed they are in the lysed cell. One possible explanation for the low contrast of the microfilament bundles in the living cells is that they are surrounded by a higher concentration of Triton X-soluble material, such as G-actin, than the microtubules. It has been shown that Triton X removes 60–70% of the total cellular protein from tissue culture cells, with actin as the major soluble component (28). Removal of G-actin and any Triton X-soluble F-actin thus would result in the great increase in contrast of the microfilament bundles we observe in the lysed cell.

In the future, we plan to expand this work by comparing the optical properties, such as phase gradients (DIC microscopy) and birefringence (polarized light microscopy), of various components of the cytoplasmic matrix in both the living and/or lysed and fixed cell to the corresponding whole microtubules. This type of study may help interpret the behaviors that the components of the cytoplasmic matrix exhibit when their functions are studied with video microscopy.

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