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Formation of Membrane Networks In Vitro by Kinesin-driven Microtubule Movement

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Abstract. Certain intracellular organelles such as the endoplasmic reticulum (Terasaki, M., L. B. Chen, and K. Fujiwara. 1986. *J. Cell Biol.* 103:1557-1568) and lysosomes (Swanson, J., A. Bushnell, and S. C. Silverstein. *Proc. Natl. Acad. Sci. USA.* 84:1921-1925) form tubular networks that are closely aligned with microtubules. Here we describe the formation of polygonal networks composed of interconnected membrane tubules that occurs when a preparation of microtubule affinity-purified squid kinesin is combined with microtubules and ATP on a glass surface. The membrane, which is a minor contaminant in the microtubule affinity-purified kinesin preparation, binds to microtubules translocating along kinesin-coated glass surfaces. Force exerted by kinesin upon the microtubule is transmitted to the membrane and a tubular extension of the membrane is produced. As the membrane tubule elongates, membrane tension exerts an

opposing force upon the translocating microtubule that can alter its direction of movement by dissociating or partially dissociating the microtubule from the kinesin-coated surface. Membrane tubules that come in contact appear to fuse with one another, and thus give rise to two-dimensional polygonal networks of tubules that have similar features to endoplasmic reticulum networks in cells. Artificial liposomes composed of dimyristoylphosphatidylcholine and yolk phosphatidylglycerol also form stable tubular structures when subjected to shear forces, but do not interact with microtubules or form polygonal networks, suggesting that such phenomena may require membrane-associated proteins. These findings indicate that kinesin generates sufficient force to form tubular membrane extensions in vitro and suggest that this microtubule-based motility protein may also be responsible for creating tubular membrane networks within cells.

THERE is increasing evidence that the organization and spatial arrangement of intracellular membrane organelles requires an intact network of microtubules and possibly microtubule-based motility (reviewed in Vale, 1987). The endoplasmic reticulum (ER)¹ and lysosomes are examples of organelles whose morphology and intracellular distribution depends upon microtubules. The ER, which is normally organized as a complex network of membrane cisternae and tubules throughout the cell (Porter, 1953; Buckley and Porter, 1975; Munro and Pelham, 1987), collapses towards the cell center after microtubules are depolymerized with nocodazole or by incubation at 4°C (Terasaki et al., 1984, 1986). Upon removal of the drug or warming the cells, microtubules reform and tubules of the ER migrate towards the periphery in close proximity to the newly polymerized microtubules (Terasaki et al., 1986). Tubular extensions of lysosomes in the macrophage cell line J-744.2 are also closely aligned with microtubules (Swanson et al., 1987).

The exact role of microtubules in the formation of membrane tubules has not been clearly established. One possibility, however, is that kinesin (Vale et al., 1985a), a microtubule-based motility protein that moves towards the plus ends of microtubules (Vale et al., 1985b), attaches to the ER or a lysosome and extends a membrane tubule as it translocates along a microtubule towards the periphery of the cell. Several observations are consistent with this hypothesis. Examination of ER or lysosomal tubules by video microscopy reveals that these processes extend by moving along linear paths at similar rates to intracellular transport of small vesicles, suggesting that the same force-generating enzymes may be involved (Lee and Chen, 1988; Swanson et al., 1987). Furthermore, crossbridges linking the ER to microtubules have been identified by electron microscopy (Franke, 1971), which have similar features to crossbridges connecting transported vesicles to microtubules in axons (Miller and Lasek, 1985).

One approach for understanding how tubular extensions of intracellular organelles are produced is to examine the formation of such structures in a reconstituted system. Here, we describe an in vitro system where kinesin-driven microtubule movement provides a driving force for the formation of

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; MAP(s), microtubule-associated protein(s).

membrane tubules and networks on a glass surface; the morphology of these networks is similar to that described for the endoplasmic reticulum *in vivo*. These results suggest that force generated by microtubule-based motility proteins such as kinesin may be responsible for the formation of tubular membrane networks within cells.

Materials and Methods

Materials

Taxol was a generous gift of Dr. Matthew Sufness of the National Cancer Institute. Octyldecyl rhodamine B (R18) was purchased from Molecular Probes, Inc., Junction City, OR. All other chemicals used in this study were purchased from the Nakarai Chemical Co., Tokyo, Japan.

Preparation of Microtubules and Kinesin

Tubulin was prepared from bovine brain by three cycles of warm and cold polymerization (Shelanski et al., 1973). Microtubule-associated protein (MAP)-free microtubules for affinity purification of kinesin were prepared by first polymerizing thrice-cycled tubulin (in microtubule assembly buffer: 80 mM Pipes, pH 6.9, 1 mM MgCl₂, 1 mM EGTA) with 20 μM taxol and 1 mM GTP for 15 min at 23°C and then removing MAPs by addition of 1 M NaCl for 15 min at 23°C. Microtubules were separated from associated proteins by centrifugation at 70,000 rpm for 15 min at 23°C in a centrifuge (model TL100; Beckman Instruments, Inc., Palo Alto, CA) and resuspended in assembly buffer with 1 mM GTP and 20 μM taxol to stabilize microtubules. For microtubule movement assays, tubulin was further purified by phosphocellulose chromatography (Weingarten et al., 1975) and was polymerized into short microtubules by the addition of 20 μM taxol and 1 mM GTP at 23°C.

Kinesin was prepared from squid (*Loligo paellii*) optic lobes that were obtained at the Marine Biological Laboratory (Woods Hole, MA) and frozen in liquid N₂. Once thawed, the following procedures were conducted at 4°C. Optic lobes were homogenized in microtubule assembly buffer containing 0.5 mM ATP, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml soy bean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml TAME (1.5:1 buffer volume to weight ratio) with 40 strokes in a Dounce homogenizer with a type A pestle (Kontes Glass Co., Vineland, NJ). The homogenate was then centrifuged for 30 min at 40,000 g, and the supernatant (S1) was centrifuged at 100,000 g for 60 min to yield a S2 supernatant. (In some instances, the supernatant [S1] was frozen and stored in liquid N₂ before proceeding to the subsequent centrifugation.) Taxol-stabilized microtubules (200 μg/ml), 0.1 mM GTP, and 15 μM taxol were added to S2, and hexokinase (20 U/ml), glucose (5 mM), and adenylyl 5'-imidodiphosphate (AMP-PNP; 2 mM) were subsequently added for 40 min at 23°C to promote kinesin attachment to microtubules. Microtubules with bound kinesin were centrifuged for 30 min at 40,000 g, and the pellet was washed with assembly buffer and then resuspended in 1:20 of the S2 volume in assembly buffer containing 10 mM MgATP, 1 mM GTP, 20 μM taxol and incubated for 1–2 h at 4°C to release kinesin from the microtubules. The microtubules were then centrifuged as before, and the supernatant containing kinesin as well as other proteins and membranes (termed microtubule affinity-purified kinesin) was collected and stored on ice. The protein concentration was generally between 300–500 μg/ml.

Membrane Network Assay and Dark Field Microscopy

Microtubule affinity-purified kinesin (8 μl) was combined with microtubules (1 μl of a 500 μg/ml solution of microtubules polymerized from phosphocellulose-purified tubulin with 20 μM taxol and 1 mM GTP) and ATP (1 μl of a 10 mM solution). 5 μl of this mixture was applied as a drop to a glass slide, and a 22 × 22 mm glass coverslip was applied directly on top. Networks, which formed on the slide surface, were sparse and generally only a few (1–20) were observed on each slide.

Microtubules and networks were examined by light microscopy using dark field illumination, as described by Horio and Hotani (1986). Images were projected onto the target of an Ikegami CTC 9000 SIT camera and were recorded using a VHS tape recorder (Panasonic NV8950). Photographs of dark field images were obtained directly from the microscope using Kodak Tri-X film and 35-mm camera mounted to a trinocular port on the microscope, or were obtained from recorded images by photographing a monitor with Pan-X film.

Preparation of Tubular Liposomes

Dimyristoylphosphatidyl choline and yolk phosphatidylglycerol (0.25 μmol each combined in 0.5 ml of 98% CHCl₃ and 2% CH₃OH) were dried down with a stream of N₂, evacuated for 60 min, and resuspended in 0.5 ml of microtubule assembly buffer to form liposomes. 1 μl of liposomes was added to the normal assay mixture described above. Tubular liposomes were formed by the shear forces generated by first pipetting the mixture several times with a pipette (model P200; Gilson Co., Inc., Worthington, OH) and then placing a coverslip on top of a 5-μl drop on a glass slide and tapping it gently.

Results

Formation of Networks on Glass Surfaces

Kinesin from squid optic lobes was prepared by incubating ATP-depleted high speed supernatants with taxol-stabilized microtubules and the nonhydrolyzable ATP analogue, AMP-PNP. Under such conditions, soluble kinesin binds to microtubules, and microtubule-kinesin complexes can be separated from the supernatant by centrifugation. Squid kinesin, which is composed of 110- and 70-kD polypeptide subunits (Vale et al., 1985a, b), was subsequently released from the microtubules with ATP and was ~25–50% pure as estimated by SDS-PAGE (Fig. 1). Many of the contaminating proteins are very likely other microtubule-binding proteins.

When microtubule affinity-purified kinesin, taxol-polymerized microtubules, and ATP were applied to a glass slide, microtubules in the solution attached to the glass and translocated along the surface, as observed by dark field microscopy. Elongated strands that were much longer (up to 100 μm long) and brighter than microtubules were also observed emerging from large amorphous objects in a few locations on the glass surface (generally between 1 and 20 per slide) (Fig. 1). Strands sometimes anastomosed with one another and formed large networks of interconnected polygons (Fig. 1). These structures, which only formed on the glass surface, were not observed in either the kinesin or the microtubule preparation alone. They formed in a dynamic fashion when the kinesin preparation, microtubules, and ATP were combined together, as will be discussed later.

Networks Are Lipid-containing Membranes

Several pieces of evidence suggest that the strands and networks observed by dark field microscopy are lipid-containing membranes. First of all, networks rapidly dissolved when exposed to a 0.5% Triton X-100-containing buffer (Fig. 2). Microtubule movement on the glass continued after the network was solubilized with detergent. Networks and processes also became fluorescently labeled after addition of a fluorescent lipid analogue (octadecyl rhodamine B [R18]) that partitions into membrane bilayers (Fig. 3). Furthermore, networks and strands swelled and developed a beaded appearance when slides were perfused with a hypotonic solution, suggesting that they are enclosed lipid bilayers (not shown). When examined by dark field microscopy adjusted to low contrast, a luminal space was observed within a few wide and short strands that emerged from the large, amorphous membranes on the glass (not shown), again suggesting that at least some of the tubular processes are composed of uni- or multilamellar membrane bilayers. A luminal space within large artificial liposomes has also been visualized by dark field microscopy (Hotani, 1984). A luminal space was

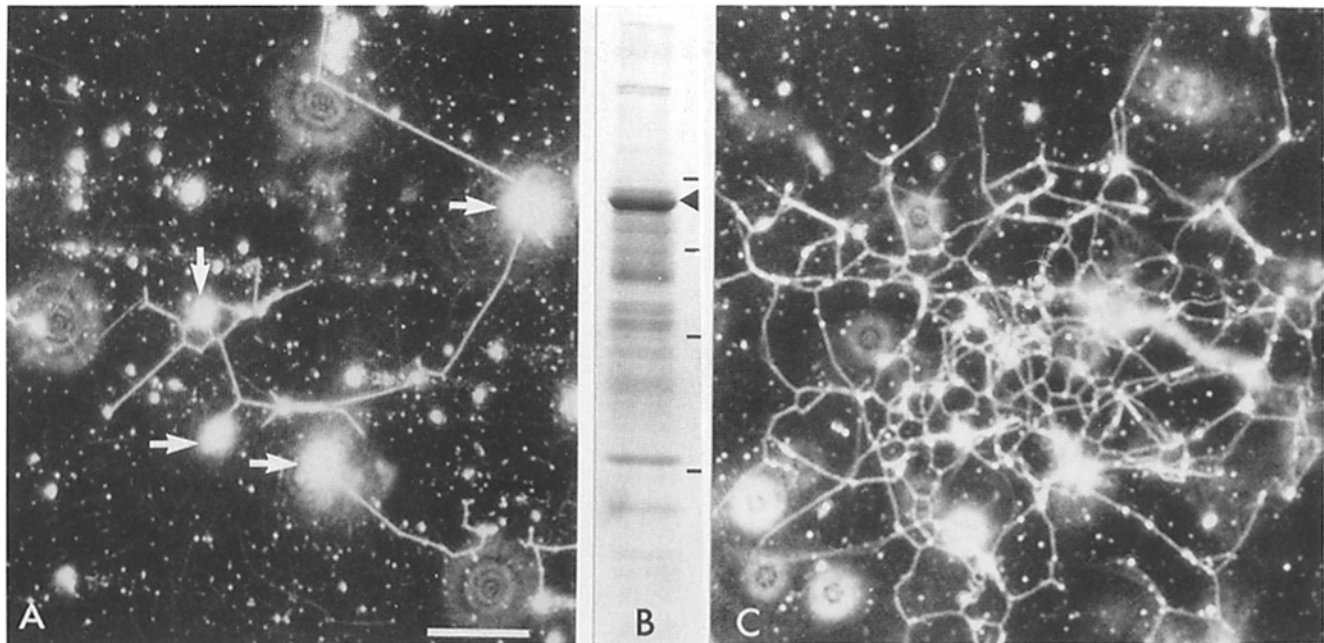


Figure 1. Examples of membrane tubules (*A*) and networks (*C*) that form when a partially purified preparation of kinesin, containing contaminating membranes, is combined with purified microtubules and ATP and placed between a glass slide and coverslip. The kinesin preparation analyzed by SDS-PAGE (7.5%) is shown in *B*. The black arrowhead indicates the position of the 110-kD subunit of kinesin, and the dashes indicate the migration positions of molecular mass markers (205, 116, 97, 66, 45 kD from top to bottom). Membrane tubules generally extend from larger membranes attached to the glass (arrows in *A*) that presumably act as reservoirs for fluid flow of lipid into the tubules as they extend by microtubule movement. Images of microtubules generate much less contrast than the membrane tubules and are barely visible as faint 1–10- μm linear elements in these photographs. A network composed of interconnected membrane tubules with threeway junctional branch points is shown in *C*. *A* and *C* are from different regions of the same coverslip. Darkfield micrograph. Bar, 10 μm .

not visible in the majority of strands, probably because their diameters were smaller than the resolution limit of our dark field microscope system ($\sim 0.4 \mu\text{m}$). The lower limit of tubule diameters is not known, although the diameter of tubules generated from artificial liposomes by mechanical forces can be as small as 10–20 nm (Hochmuth and Evans, 1982).

The network-forming membrane appears to be a contaminant in the microtubule affinity-purified kinesin preparation (five of seven preparations of kinesin from squid optic lobe contained membranes capable of forming strands or networks). Treatment of the kinesin fraction with Triton X-100 (0.5%) or phospholipase A₂ eliminated network formation but did not affect kinesin-induced microtubule movement (Table I). Kinesin that was further purified by gel filtration chromatography (Bio-Gel A5m; Bio-Rad Laboratories, Richmond, CA), which separates contaminating membranes from the kinesin, also did not form networks in the motility assay. The cellular origin of the network-forming membrane is unknown. The majority (>80%) of the detergent-soluble objects on the glass surface, however, did not form tubules or networks, suggesting that the contaminating membrane may be heterogeneous and that only a subset of the total membrane may be capable of forming such structures.

Membrane Tubule Formation Requires Microtubule Movement

Membrane tubules form as the result of interactions between microtubules translocating on kinesin-coated glass and large

membranes attached to the same glass surface. Inhibition of kinesin-driven microtubule movement by AMP-PNP or high concentrations of vanadate (100 μM) eliminated network and process formation (Table I). Microtubule movement and network formation was also inhibited by treating glass slides with polylysine (Table I).

Network formation could be prevented or diminished by treating the glass surface with a siliconizing compound or by decreasing the surface to volume ratio by placing spacers (fragments of a No. 1 glass coverslip) between the coverslip and slide. Under such conditions, microtubule translocation occurred but few large membranes were firmly attached to the glass. Such results suggest that membranes attachment to the glass surface is required for tubule extension, although it is possible that these treatments have additional effects besides decreasing membrane attachment to the surface.

Dynamics of Network Formation

Membrane tubules and networks were observed ~ 5 –15 min after adding microtubule affinity-purified kinesin, microtubules, and ATP to the slide. Observation by video microscopy revealed that when translocating microtubules came in contact with a large membrane, they sometimes extended a membrane tubule as they continued to move along the glass (Fig. 4). The membrane tubules appeared to elongate by a flow of lipids from the larger membranes on the glass (termed “membrane reservoirs”). Microtubules could also attach to a preexisting membrane tubule and extend a new

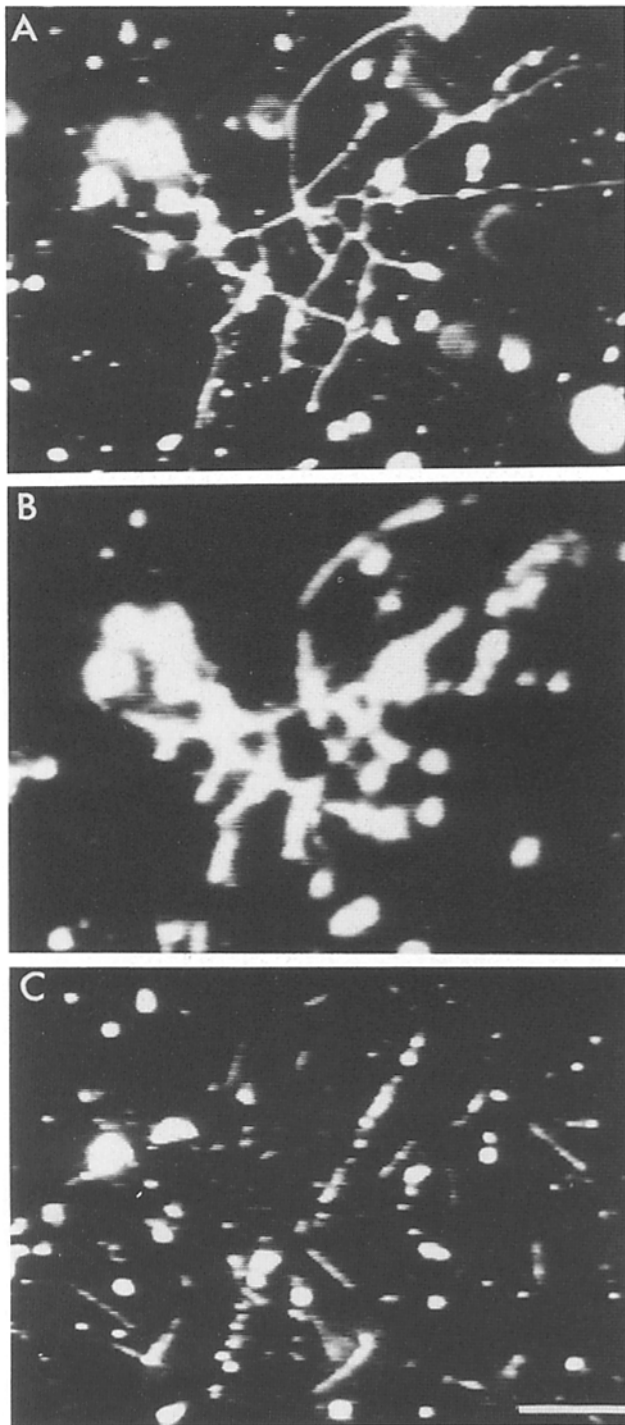


Figure 2. Solubilization of a membrane network with Triton X-100. This sequence from video tape shows a membrane network dissolving when Triton X-100 (0.5% in microtubule assembly buffer with 1 mM ATP) is perfused in from one side of a coverslip while fluid is withdrawn from the opposite side with filter paper. (A) The membrane network before perfusion; (B) an increase in contrast is observed as the detergent-containing buffer reaches the network during perfusion; (C) after perfusion, the network dissolves and the microtubules, the rod-shaped objects, continue to move along the coverslip. Other objects that remain after detergent perfusion are either small particles in the buffer or imperfections on the glass slide that are visible by dark field microscopy. Bar, 10 μ m.

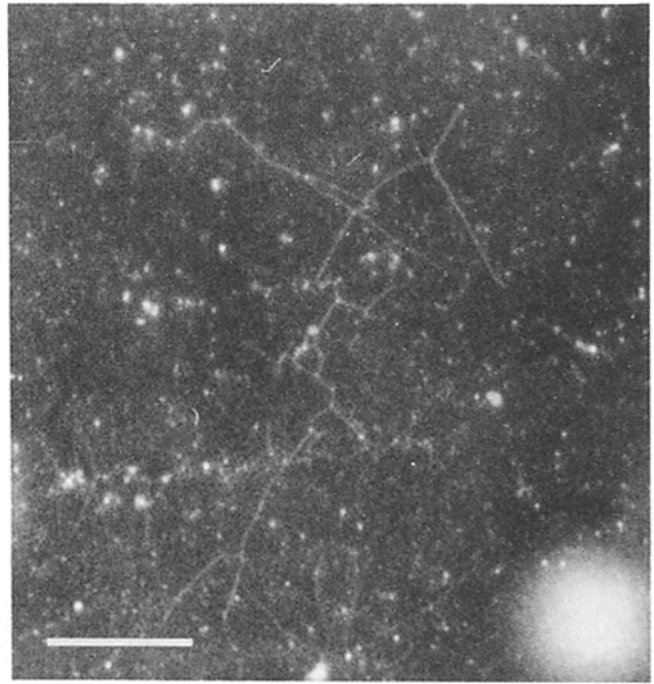


Figure 3. Fluorescence photomicrograph showing tubules stained with octadecyl rhodamine B (R18). 1 μ l of a 100 nM solution of R18 in DMSO was added to the assay mixture described in Materials and Methods. Tubular processes that formed on the coverslip were fluorescently labeled when examined by epifluorescence using a rhodamine filter set. Because of nonspecific adsorption of R18 to the glass, the background fluorescence is high and the image contrast of these tubules is not as great as images of tubules obtained by dark field microscopy. Bar, 10 μ m.

tubule branch. Membrane tubules eventually dissociated from translocating microtubules and elastically recoiled and assimilated with the membrane reservoir. In some instances, however, interactions between the tubule and the glass surface prevented the tubule from recoiling after it dissociated from the microtubule, and the membrane maintained its tubular configuration on the glass surface (see Fig. 1 A).

Linear tubules were converted into a two-dimensional network of interconnected polygons (Figs. 1, 2, and 4) as the result of encounters and apparent fusion events between elongating membrane tubules. The following observations suggest that fusion can occur between tubules. Fig. 5 shows a translocating microtubule that extended a membrane tubule across another tubule. Initially, the two tubules were linear and crossed one another at single point, but within a few seconds a rearrangement took place that produced two junctions, each with three tubule branches. The translocating microtubule continued to extend the membrane, but after the rearrangement, it only exerted tension upon and extended the branch to which it was attached. Thus, after the rearrangement, the linear membrane tubule was divided into two branches, each capable of moving independently of the other. Such observations suggest that fusion occurred at the intersection between the two membrane tubules. The polygonal networks also did not dissociate or become disconnected into linear tubules when subjected to shear forces introduced

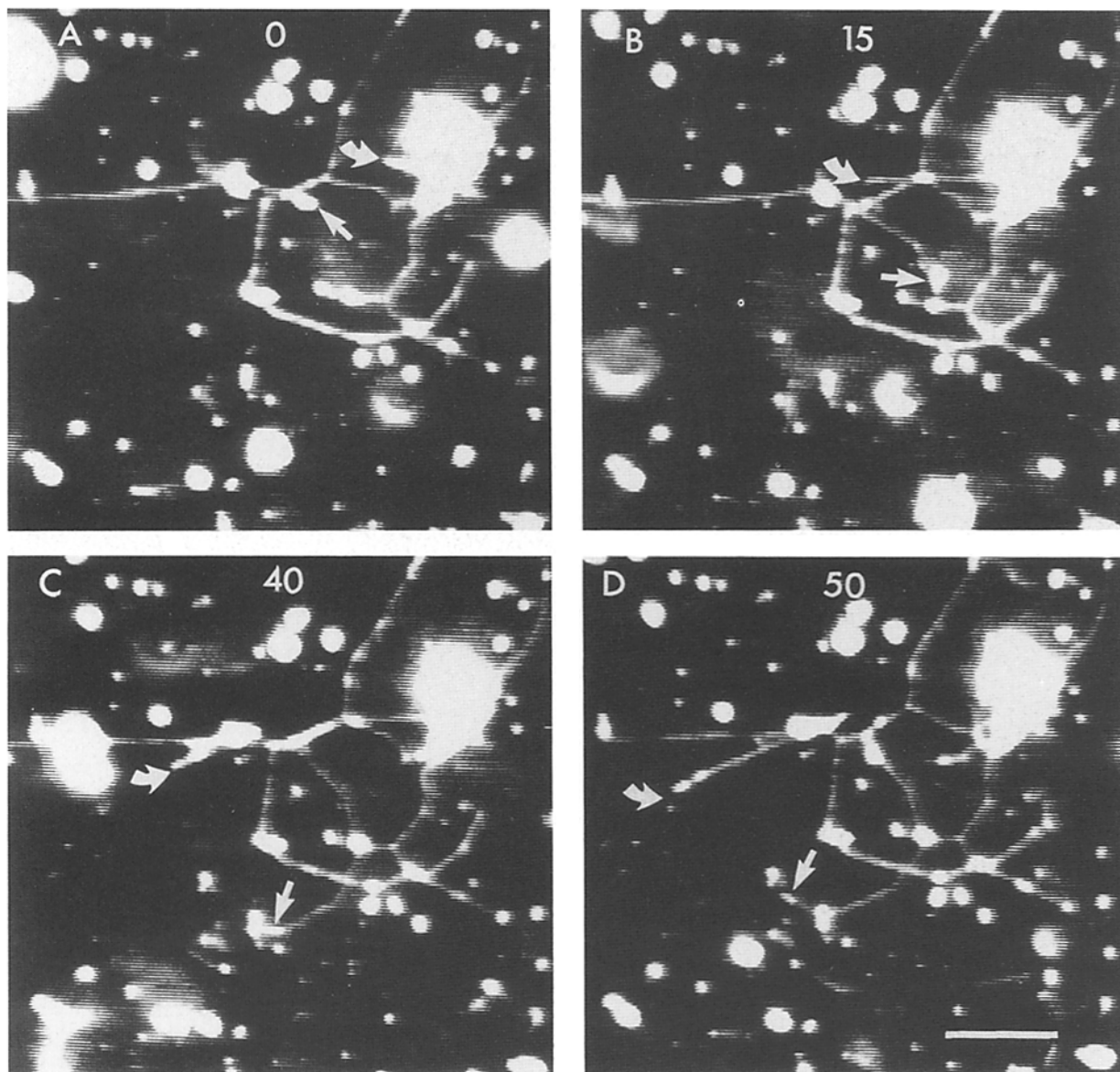


Figure 4. Formation of membrane tubules by microtubule movement. This sequence shows two short taxol-polymerized microtubules (their respective positions are indicated by the straight and curved arrows) that extend membrane as they translocate along the kinesin-coated glass surface. Elapsed time in seconds is shown at the top of each panel. Bar, 10 μm .

by buffer perfusion, again suggesting that they are composed of a continuous network of tubules and not merely overlapping tubules. Fusion and subsequent rearrangements of tubules produced stable structures that did not recoil and assimilate with membrane reservoirs. Formation of new membrane tubules were infrequently observed 40–60 min after the mixture was applied to the glass slide, however, the polygonal networks of membrane tubules remained intact for as long as 1–2 h, after which time they gradually disintegrated.

Membrane tubules exhibited many elastic and fluid properties that are also characteristic of membrane extensions or “tethers” produced by mechanical forces from liposomes or red blood cell membranes (Vaugh, 1982*a, b*; Hotani, 1984; Hochmuth et al., 1979). The rapid recoil of membrane tu-

bules after they dissociate from microtubules suggests that they are highly elastic and deformable structures. Furthermore, junctions between tubules can undergo lateral movements and rearrangements, phenomena which are also consistent with the fluid properties of membrane bilayers (Figs. 5 and 6). Lateral movement of a junctional branch point between membrane tubules in a polygonal network can lead to the assimilation of one tubule with another and hence to the closure and disappearance of a polygon. A similar phenomenon of “ring closure” of ER polygons in living cells has been described by Lee and Chen (1988).

As membrane tubules elongate, they exert an opposing force upon translocating microtubules that can alter the direction of microtubule movement. Microtubules, which normally translocate in a linear fashion on kinesin-coated

Table I. Effect of Agents on Microtubule Movement and Network Formation

Agent	Microtubule movement	Networks
None	+	+
Vanadate		
10 μ M	+	+
100 μ M	-	-
AMP-PNP (10 mM)	-	-
NEM (1 mM)	+	+
Triton X-100 (0.5%)	+	-
Phospholipase A ₂	+	-
Polylysine-coated glass	-/+	-
Silane-coated glass	+	-

Sodium orthovanadate, AMP-PNP, and Triton X-100 were added directly to the assay mixture immediately before being placed on the slide. Phospholipase A₂ (100 U/ml) and NEM were incubated with the microtubule affinity-purified kinesin for 15 min at 23°C. Unreacted NEM was inactivated with 10 mM DTT before combining the kinesin mixture with microtubules. Effects of polylysine and silane coating of the glass surface were analyzed by treating slides and coverslips with 100 μ g/ml polylysine or a silicizing compound (Surfacil; Pierce Chemical Co., Rockford, IL) for 30 min at 23°C and then washing exhaustively with distilled water. The presence (+) or absence (-) of microtubule movement along glass and the formation of processes and networks was then assayed. -/+ indicates that some movement was observed, although significantly reduced compared with the control.

glass surfaces, often followed curved or circular paths when tethered to a membrane tubule (Fig. 4). In some instances, microtubules even reversed their direction of movement after extending a membrane tubule a certain distance (Fig. 6). The force exerted by the membrane appeared to alter the direction of movement by causing the microtubule either to dissociate from the kinesin-coated glass surface or to slightly change its axial orientation while remaining bound to a kinesin. In some instances, the membrane-tethered microtubule abruptly dissociated and completely reversed its orientation by 180°. A microtubule sometimes extended and then retracted a membrane tubule (after reversing its direction of movement) several times before the membrane eventually dissociated. Microtubules in the process of extending or retracting membrane tubules moved at similar velocities to unattached microtubules translocating on the same glass slide (Table II). Measurement of displacement distances at 1-2-s time intervals indicated that the velocity of microtubule movement did not progressively decrease as the membrane tubule extended (not shown). Thus membrane attachment to a microtubule is capable of changing the direction of microtubule movement on glass, while producing relatively little effect upon the velocity of microtubule movement. We interpret these results to indicate that the elastic restoring force of the membrane dissociates the microtubule from kinesin molecules on the glass before it exceeds the kinesin-induced force and stalls movement.

Formation of Tubules from Artificial Liposomes

Liposomes, composed of an equimolar mixture of dimyristoylphosphatidyl choline and egg phosphatidylglycerol, form elongated tubules (often several hundred microns long) in the absence of microtubules and kinesin when subjected to shear forces produced by fluid flow (Fig. 7). Like the membrane tubules in the kinesin preparation, these artificial liposome tubules formed branches with threeway junctions and were

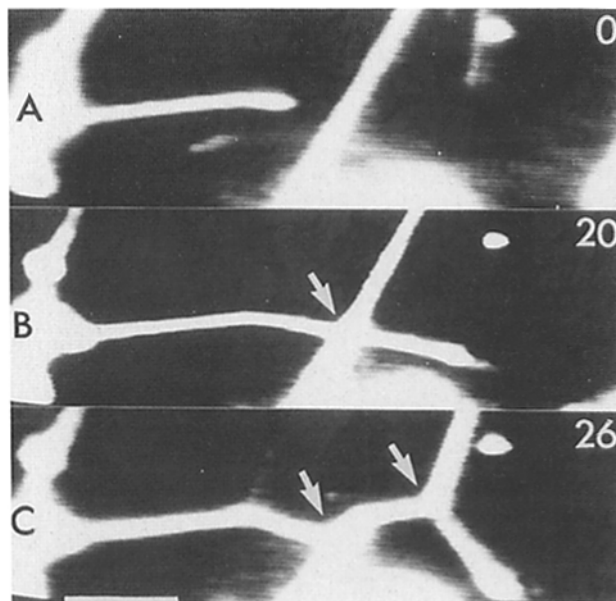


Figure 5. Apparent fusion of two membrane tubules. This sequence shows a membrane tubule (*left*) being extended by a microtubule translocating from left to right. In *B*, the microtubule intersects a second tubule (*arrow*). In *C*, two junctional branch points have formed from the initial point of intersection. Tension created by the translocating microtubule causes the branch point on the right to move laterally away from the other branch point whose position has not changed significantly. Such behavior of tubules is most consistent with a fusion event occurring between the two membrane tubules at a time in between *B* and *C*. The time elapsed (in seconds) is indicated in the upper right of each panel. Bar, 5 μ m.

also stable for >1 h at room temperature. A junction between two liposome tubules was also capable of lateral movement along one of the membrane tubules. Artificial liposomes, however, did not form networks of closed polygons and, when combined with kinesin and microtubules, they did not attach to microtubules moving along the glass surface. Although the lipid composition of these liposomes is different from most biological membranes, these observations nonetheless suggest the possibility that membrane-associated proteins may be involved in mediating interactions between the squid optic lobe membranes and microtubules.

Discussion

In this study, the formation of membrane tubules and networks has been examined in an *in vitro* system. Force is required to extend a membrane into a tubular configuration. Shear forces produced by osmotic shock, mechanical deformation, or bulk fluid flow (Hotani, 1984; Hochmuth and Evans, 1982; Waugh, 1982*a, b*), or forces associated with tubulin polymerization into microtubules within liposomes (Hotani, H., unpublished observations) are all capable of elongating membrane into tubules or tethers. Such forces are not responsible for producing membrane tubules in our system, however, and are unlikely to be involved in forming large tubular networks such as the ER in living cells. In the system described here, the force responsible for forming membrane tubules and networks is generated by kinesin. Tu-

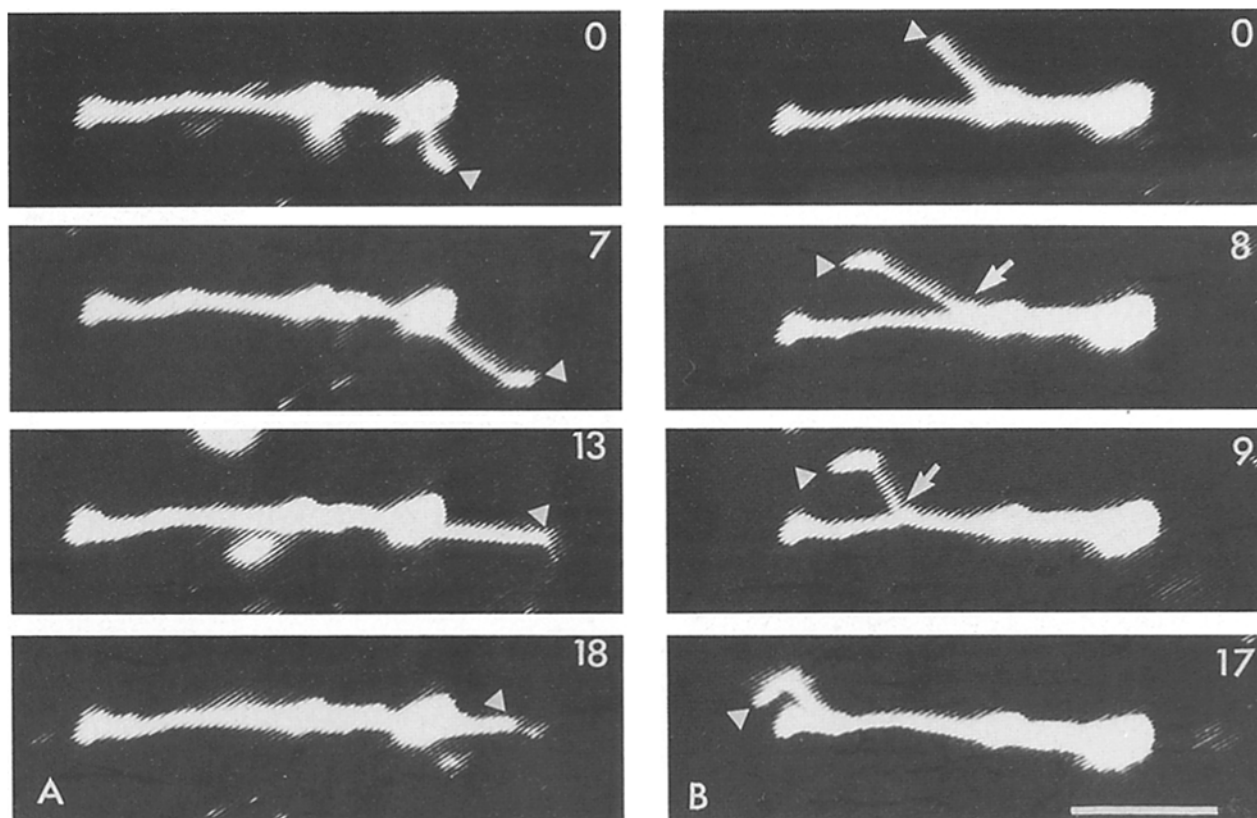


Figure 6. Elastic properties of membrane tubules. *A* shows the effects of membrane attachment on the path of microtubule translocation. A short microtubule at the end of a membrane tubule (*arrowhead*) extends the membrane towards the lower right. As a consequence of its attachment to the membrane, the microtubule does not translocate along a straight path, but instead follows a curved path and gradually reverses its direction and translocates back to the larger membrane reservoir. *B* illustrates the rapid lateral mobility of junctional intersections between membrane tubules. This sequence shows a microtubule (*arrowhead*) that moves from right to left along the glass surface whilst attached to a membrane. The branch point of the two membrane tubules (*arrow*) moves rapidly between 8- and 9-s time points. The time elapsed (in seconds) is indicated in the upper right corner of each photograph. Bar, 10 μm .

bule formation involves: (a) attachment of membranes to the glass; (b) movement of microtubules along the kinesin-coated glass coverslip; and (c) formation of a strong association between membranes and microtubules that enables kinesin-induced force to be transmitted to the membrane.

Table II. Effect of Membrane Attachment on the Velocity of Microtubule Movement

Sample	Velocity $\mu\text{m/s}$
(a) Microtubule attached to membrane (membrane tubule extending)	0.43 ± 0.05
(b) Microtubule attached to membrane (membrane tubule retracting)	0.51 ± 0.08
(c) Microtubule unattached to membrane	0.51 ± 0.04

Microtubules chosen for velocity analysis were those that (a) extended a membrane tubule, and then reversed orientation by 180° (by temporarily dissociating from the glass) and then (b) retracted the membrane tubule while translocating back to the membrane reservoir ($n = 12$ microtubules). (c) Mean velocities and standard deviations of microtubules that were not attached to membranes but which were translocating on the same kinesin-coated glass surface are shown ($n = 40$).

The magnitude of the kinesin-induced force imparted to the membrane is presently unknown.

The morphologies of the *in vitro* tubular membrane networks bear a striking resemblance to networks of ER tubules in cells (Buckley and Porter, 1975; Terasaki et al., 1984, 1986; Munro and Pelham, 1987). The *in vitro* and *in vivo* networks also exhibit many similar dynamic properties. The extension of a new ER (Lee and Chen, 1988) or lysosomal tubule branch (Swanson et al., 1987), requires the application of force at a single point on the membrane, while the conversion of linear tubules into polygonal networks appears to involve fusions between contacting membrane tubules. Other dynamic properties of ER tubules such as sliding (movement of an ER junction along a tubule) and ring closure (contraction and disappearance of polygons by sliding of an ER junction) (Lee and Chen, 1988) have also been observed in this *in vitro* system. Such movements appear to achieve a minimum energy configuration of the lipid-containing tubules in response to changes in tension on the tubule or the network.

Although tubules in polygonal networks frequently undergo rearrangements, the polygonal configuration of the *in vitro* networks are maintained for 1 h or more even in the absence of a continuous association with microtubules or con-

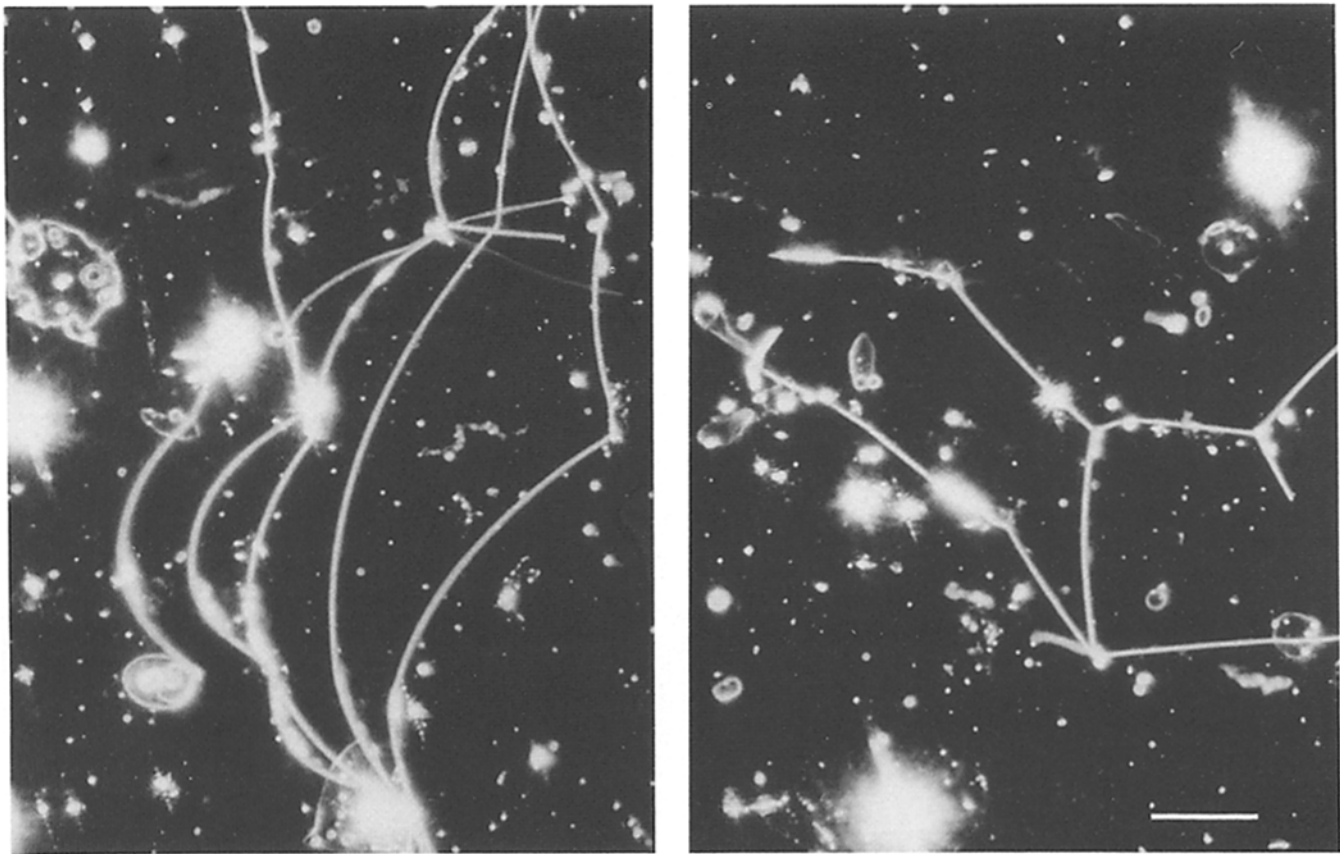


Figure 7. Liposomes composed of dimyristoylphosphatidyl choline and yolk phosphatidylglycerol form elongated tubules when subjected to shear forces. Tubular liposomes were prepared as described in Materials and Methods and do not require microtubules for their formation. These tubular liposomes form threeway branches, but do not form networks of closed polygons. Bar, 8 μm .

tact throughout their length with the glass surface. Observations of individual ER polygons in living cells indicate that they are also stable for at least 30 min (Lee and Chen, 1988). Vesicularization of ER tubules, which has been reported to occur during mitosis (Zeligs and Wollman, 1979), therefore, may not be due to the inherent instability of tubular membrane networks after cytoplasmic microtubules depolymerize at the onset of mitosis, but may involve a specific perturbation analogous to the breakdown of the nuclear envelope (Newport and Spann, 1987) or the fragmentation of the Golgi apparatus (Lucocq and Warren, 1987).

The formation of membrane tubules and tubular networks has also been recently observed in crude extracts from chick fibroblasts (Debora and Sheetz, 1988) and the alga *Nitella* (Kachar and Reese, 1988). Unlike the system described here where membrane tubules are extended by microtubules translocating along the glass substrate, microtubules or actin cables in these crude extracts are stationary and membrane tubules are formed by the active movement of the membrane along the microtubule (chick fibroblasts) or actin cable (*Nitella*). The membrane-associated force-generating enzymes responsible for tubular network formation in the extracts have not been conclusively identified, although kinesin and myosin are the most likely candidates for the microtubule and actin-based transport motors that pull membranes along microtubules in fibroblast extracts and along actin in the *Nitella* extracts, respectively.

The formation of membrane tubules and networks *in vivo* probably also occurs by active transport of membrane along stationary cytoskeletal polymers, similar to that described in the crude extracts, and not by the active movement of cytoskeletal polymers. Recent microscopic studies of living cells, for example, show that ER tubules (Lee and Chen, 1988) extend along linear paths in a similar fashion to microtubule-based movement of small vesicular organelles. Since the majority of microtubules are anchored at their minus ends to the centrosome, such observations are most readily explained by active transport of the membrane along microtubules driven by force-generating motors attached to the membrane surface. Although the exact mechanism of membrane tubule extension in our system differs from that described in these crude extracts and possibly *in vivo*, the morphologies and dynamic properties of networks are nonetheless similar to one another. The features common to these *in vitro* systems that appear to be required for the formation of tubular membrane networks are: (a) a mechanism of force-generation for extending membrane into tubules; and (b) membrane fusion which allows tubules to form closed polygonal networks.

Although the preparation described here has been useful for examining the process of membrane tubule formation in the context of the well-defined kinesin-microtubule motility assay, the vanishingly small quantities of membrane obtained have presented substantial difficulties in biochemically char-

acterizing the network-forming membrane and the microtubule-membrane interaction. A more reliable and larger scale purification of network-forming membranes will have to be developed to allow such biochemical work to proceed. This study as well as others (Suprenant and Dentler, 1982), however, suggests that microtubule affinity, which has provided a successful strategy for the purification of MAPs and motility proteins such as kinesin, may also provide a useful step in purifying intracellular organelles that interact with microtubules.

In conclusion, the results presented here using purified kinesin, as well as those by Debora and Sheetz (1988) and Kachar and Reese (1988) using motor-containing cell extracts indicate that microtubule and actin-based motility proteins generate sufficient force to extend membrane tubules in vitro. The dynamic properties of the in vitro tubular membrane networks formed by such processes also provide insight into the formation and behavior of the ER in living cells (Lee and Chen, 1988). The formation of threeway tubule junctions as well as the rearrangement and sliding of tubule junctions, which have been observed with the ER in vivo as well as with the in vitro membrane networks and artificial tubular liposomes, appear to be elastic responses of lipid-containing membranes to tension. The microtubule-based extension of membrane tubules and subsequent tubule-tubule fusion, on the other hand, may require membrane-associated protein to serve as anchoring points for force generation and possibly for facilitating fusion between membrane tubules. Thus, our observations suggest that the morphological appearance of the ER in cells and of tubular membrane networks in vitro is due to the application of kinesin-induced force to a membrane, the fusion between membrane tubules, and the reorganization of membrane tubules due to their intrinsic elastic and fluid properties. The direction of ER migration from the cell center to the periphery (Terasaki et al., 1986) is also consistent with a kinesin-driven rather than a dynein-driven process (Vale, 1987).

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References

Buckley, I. K., and K. R. Porter. 1975. Electron microscopy of critical point dried whole cultured cells. *J. Microsc.* 104:107-120.

- Debora, S. L., and M. P. Sheetz. 1988. Microtubule dependent formation of a tubular-vesicular network with characteristics of the endoplasmic reticulum from cultured cell extracts. *Cell*. 54:27-35.
- Franke, W. W. 1971. Cytoplasmic microtubules linked to endoplasmic reticulum with cross-bridges. *Exp. Cell Res.* 141:139-147.
- Hochmuth, R. M., and E. A. Evans. 1982. Extensional flow of erythrocyte membrane from cell body to elastic tether. I. Analysis. *Biophys. J.* 39:71-81.
- Hochmuth, R. M., P. R. Worthy, and E. A. Evans. 1979. Red cell extensional recovery and the determination of membrane viscosity. *Biophys. J.* 26:101-114.
- Horio, T., and H. Hotani. 1986. Visualization of the dynamic instability of individual microtubules by dark-field microscopy. *Nature (Lond.)*. 321:605-607.
- Hotani, H. 1984. Transformation pathways of liposomes. *J. Mol. Biol.* 178:113-120.
- Kachar, B., and T. S. Reese. 1988. The mechanism of cytoplasmic streaming in characaeal cells: sliding of endoplasmic reticulum along actin filaments. *J. Cell Biol.* 106:1545-1552.
- Lee, C. H., and L. B. Chen. 1988. Behavior of endoplasmic reticulum in living cells. *Cell*. 54:37-46.
- Lucocq, J. M., and G. Warren. 1987. Fragmentation and partitioning of the Golgi apparatus during mitosis in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3239-3246.
- Miller, R. H., and R. J. Lasek. 1985. Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181-2193.
- Munro, S., and H. R. B. Pelham. 1987. A c-terminal signal prevents secretion of luminal ER proteins. *Cell*. 48:899-907.
- Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell*. 48:219-230.
- Porter, K. R. 1953. Observations on a submicroscopic basophilic component of the cytoplasm. *J. Exp. Med.* 97:727-750.
- Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA*. 70:765-768.
- Suprenant, K. A., and W. L. Dentler. 1982. Association between endocrine pancreatic secretory granules and in-vitro-assembled microtubules is dependent upon microtubule-associated proteins. *J. Cell Biol.* 93:164-175.
- Swanson, J., A. Bushnell, and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depend on the integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA*. 84:1921-1925.
- Terasaki, M., L. B. Chen, and K. Fujiwara. 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* 103:1557-1568.
- Terasaki, M., J. Song, J. R. Wong, M. J. Weiss, and L. B. Chen. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde-fixed cells with fluorescent dyes. *Cell*. 38:101-108.
- Vale, R. D. 1987. Intracellular transport using microtubule-based motors. *Annu. Rev. Cell Biol.* 3:347-378.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985a. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell*. 42:39-50.
- Vale, R. D., B. J. Schnapp, T. Mitchison, E. Steuer, T. S. Reese, and M. P. Sheetz. 1985b. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell*. 43:623-632.
- Waugh, R. E. 1982a. Surface viscosity measurements from large bilayer vesicle tether formation. I. Analysis. *Biophys. J.* 38:15-18.
- Waugh, R. E. 1982b. Surface viscosity measurements from large bilayer vesicle tether formation. II. Experiments. *Biophys. J.* 38:19-28.
- Weingarten, M. D., A. H. Lockwood, S.-C. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA*. 72:1858-1862.
- Zeligs, J. D., and S. H. Wollman. 1979. Mitosis in rat thyroid epithelial cells in vitro. *J. Ultrastruct. Res.* 66:53-77.