Journal of Supramolecular Structure 5:531 (383)–563 (415) (1976)

Two General Classes of Cytoplasmic Actin Filaments in Tissue Culture Cells: The Role of Tropomyosin

Elias Lazarides

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309

In the assembly of actin filaments that takes place during the spreading of a population of human lung cells, after trypsin detachment off the substratum and replating, tropomyosin exhibits a considerable lag in its association with the newly forming filament bundles; it begins to associate with them during the later stages of cell spreading as the actin filament bundles normally seen in interphase cells begin to organize. This lag is evident in a number of cell types that are spreading onto a substratum; it does not appear to be due to a selective degradation of this molecule during rounding up of the cells, since tropomyosin associates with the actin filament bundles after this lag even under conditions where the protein synthetic activity of the cell is inhibited to more than 95% by cycloheximide. The preferential binding of tropomyosin to fully assembled filament bundles but not to newly formed bundles of actin filaments suggests therefore the existence of two classes of actin filaments: those that bind tropomyosin and those that do not. This selective localization of tropomyosin on actin filaments was further pursued by examining the localization of this molecule in membrane ruffles. The immunofluorescent results indicate that ruffling is an actin-filament-dependent, microtubule-independent phenomenon. Tropomyosin is absent from membrane ruffles under a variety of circumstances where ruffling is expressed and, more generally, from any other cellular activity where actin filaments are expected to be in a dynamic state of reorganization or are required to be in a flexible configuration. It is concluded that in tissue culture cells tropomyosin binds preferentially to actin filaments involved in structural support to confer rigidity upon them as well as aid them in maintaining a stretched phenotype. The absence of tropomyosin from certain motile phenomena where actin filaments are involved indicates that these classes of actin filaments are regulated by cytoplasmic mechanisms distinct from that by which tropomyosin (and troponin) mediates contractility in skeletal muscle; it opens the possibility that different types of actin filaments engaged in different cellular motile phenomena in tissue culture cells may be regulated by a host of coexisting regulatory mechanisms, some as yet undetermined.

Key words: α-actinin, microtubules, membrane ruffles, cell spreading

Elias Lazarides is now at the Division of Biology, California Institute of Technology, Pasadena, CA 91125.

INTRODUCTION

There is compelling evidence today that actin purified from a variety of nonmuscle cells behaves by a number of physiological and biochemical criteria very similarly to skeletal muscle actin (1, 2). This evidence, in conjunction with the ability of actin filaments to interact with muscle heavy meromyosin either in vitro or in situ (2, 3), has prompted many investigators to assume that most of the motile phenomena observed in nonmuscle cells are mediated by regulatory mechanisms similar to those by which actin and myosin and their accessory regulatory proteins mediate movement in muscle. This idea has been reinforced by the wealth of information that indicates that molecules similar to those of skeletal muscle myosin, α-actinin, tropomyosin, and perhaps troponin can be found in nonmuscle cells (4–8, 52). The coexistence, however, of these molecules in both muscle and nonmuscle cells does not necessitate a functional equivalence, especially if one considers that muscle performs a relatively rigid form of movement that is unidirectional and highly specialized. On the other hand it is quite plausible that some forms of nonmuscle cell motility can be mediated by a similar mechanism as is probably the case in platelet contraction (9), the movement of the microvilli in the cells of the intestinal epithelium (10), or ameboid motility (11). In the case of the epithelial, endothelial, or fibroblastic cell types that are grown in tissue culture, the situation is more complicated. Tissue culture cells are highly plastic systems that undergo a number of morphological transformations both in response to their own cell cycle and in response to a variety of external stimuli. The cytoplasm of these cells is permeated by elaborate arrays of actin filaments and microtubules that respond coordinately or independently to such plastic transformations. In particular the intricate network of actin filament bundles that is seen in fully spread-out interphase cells presumably aids the cell in maintaining its stretched phenotype (stress fibers) (12). Actin filaments, however, are also found intimately associated with the cortex of the cell (13), microvilli and microspikes (14), membrane ruffles (15), the contractile ring (16), and the mitotic spindle (17, 18). Some of these structures are also known to contain microtubules, and some sort of coordinate temporary interaction between these two structural systems is a probable one. It is not known, however, if the actin molecules that participate in all these types of movement or structural support are functionally and chemically identical and are regulated by subtle cytoplasmic controls imposed on actin, or whether small chemical modifications of the actin molecule itself are sufficient to alter its properties and induce the molecule to shuttle between different modes of activity. These questions are usually difficult to approach biochemically unless the particular structure can be isolated. Direct biochemical isolation of actin yields only a small subpopulation of molecules, which need not necessarily retain the full spectrum of properties that actin has in the cell.

The availability of antibodies to actin and to other major cytoplasmic structural proteins has enabled us to use indirect immunofluorescence to follow the distribution of these molecules under a variety of cellular conditions (19, 47). Since indirect immunofluorescence allows the screening of a large number of cells, it is feasible to learn about the function of actin and their accessory proteins not only by following their temporal interaction with each other, but also by studying any preferential interaction of the actin-associated proteins with actin filaments in different types of cell motile phenomena where these filaments are involved.

In the past we have concentrated our study on the interaction of two molecules – α-actinin and tropomyosin – within the stress fibers seen in fully spread-out cells (5, 20). α-Actinin was chosen because it is found in the Z line of skeletal muscle and directly or

indirectly participates in the organization of actin filaments in a sarcomere (21). It was similarly assumed that α-actinin might interact with cellular membranes and thus produce a muscle-like Z line through which actin filaments could attach to membranes. This was shown to be the case, and α-actinin was found to be intimately associated with actin filaments in a highly ordered fashion not only along the stress fibers, but also in areas where actin filaments come into close proximity with the membrane, such as areas of contact between two cells or areas of adhesion of the plasma membrane to the underlying substratum (5). Tropomyosin was chosen because it is one of the most highly conserved regulatory molecules in evolution and it is found associated with the actin filaments in a variety of types of lower and higher eukaryotic muscles (22). In fully spread-out nonmuscle cells grown in tissue culture, tropomyosin is found intimately associated with the actin filament bundles (stress fibers) in an ordered, periodic manner, reminiscent of its association with the actin filaments within a sarcomere (20). The role, other than structural, that stress fibers play in nonmuscle cell motility is unknown. Stress fibers are mostly but not exclusively found associated with the cytoplasmic part of the plasma membrane that makes contact to the substratum (23), and they reflect the ability of actin to exist in situ in highly organized, almost paracrystalline arrays of laterally aggregated filaments. It was reasoned that any changes in the association of tropomyosin with either the stress fibers or with the actin filaments found elsewhere in the cell might reflect not only the role that tropomyosin plays in the structure and function of actin filaments, but also important regulatory differences between different sets of actin filaments found associated with different cellular processes. This idea was reinforced by the recurrent observation that tropomyosin showed a considerable lag in its association with actin filament bundles during the organization of the latter when a cell was spreading onto a glass substratum either after trypsin detachment and replating or after mitosis (24). The possibility that this lag reflected a selective degradation of tropomyosin and the need of its resynthesis before it was present in sufficient amounts to interact with the stress fibers was excluded by the observation that tropomyosin could be seen to associate with the stress fibers after the lag even when more than 95% of the biosynthetic activity of the cell was inhibited with cycloheximide (unpublished observation). The possibility therefore arose that this lag represented a specific cellular function of the molecule and reflected a state of the newly assembling actin filaments that distinguished it from their state in fully assembled stress fibers with respect to their ability to interact with tropomyosin. To gain more insight into this possibility, I have chosen to compare the association of tropomyosin in fully assembled stress fibers with the association of this molecule with actin filaments in membrane ruffles as well as to consider further the lag of the association of tropomyosin with newly forming actin filament bundles in spreading cells. The immunofluorescent localization of this molecule indicates that tropomyosin is minimally present or most of the time absent from areas of active membrane ruffling, or when actin filaments begin to assemble into stress fibers. In both of these cases actin filaments are expected to be in a continuous state of reorganization, of change in orientation as well as change in their state of polymerization or lateral aggregation. Tropomyosin is found associated with the actin filaments only after these filaments are laterally aggregated and have already formed stress fibers. I conclude therefore that in nonmuscle cells grown in tissue culture, tropomyosin differentiates between highly "plastic" forms of actin filaments and highly organized structural forms of these filaments. It selectively binds to the latter type, presumably to confer rigidity upon these filaments as well as aid them in maintaining a highly ordered and stretched phenotype. In this manner tropomyosin is seen to play the role of a "negative modulator" of motility whereby its absence reflects the ability of actin filaments

to be engaged in "plastic" and highly dynamic forms of movement, while its presence may confer structural rigidity upon these filaments engaged either in structural support (stress) or in unidirectional, subtle forms of movement. The absence of tropomyosin from certain cellular motile phenomena where actin filaments are the principal structural component like membrane ruffles, strongly indicates that these classes of actin filaments are regulated by cytoplasmic mechanisms, distinct from that by which tropomyosin (and troponin) mediates contractility in skeletal muscle. These results open the possibility that the involvement of actin filaments in different cellular processes might be regulated by a host of coexisting regulatory mechanisms, most of which are yet to be revealed.

METHODS

Cell Culture

The cells used in this work were the myoblast cell line L6 initially isolated and characterized by Yaffe (26). When passaged for a number of generations in culture, the majority of these cells exhibit extensive membrane ruffling both at the cell margin and/or the surface of the cell. Normally the cells exhibit an array of stress fibers that are usually minimally present in the cells that show extensive membrane ruffling. The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% calf serum. The human lung cell culture W138 (passage 26) was maintained in DMEM supplemented with 10% fetal calf serum. The conditions of trypsinization and replating have been described previously (25). The cells were plated at a density of approximately 15,000 cells per 12 mm coverslip.

Antibody Preparation

The antibodies used in the present work were prepared against a) chicken smooth muscle (gizzard) actin purified to homogeneity by preparative sodium dodecyl sulfate (SDS) slab gel electrophoresis prior to immunization; b) chicken skeletal muscle tropomyosin; and c) porcine skeletal muscle α-actinin. A detailed characterization of these antibodies has been presented previously (5, 19, 20, 25). Antibodies to tubulin were prepared against highly purified porcine brain tubulin (kindly provided by Drs. D. Murphy and G. G. Borisy, Laboratory of Molecular Biology, University of Wisconsin), which was further purified away from any minor contaminating proteins of different molecular weights by preparative SDS slab gel electrophoresis prior to immunization. This method is similar to that used for the preparation of antibodies to actin (20) with minor modifications and is presented here in detail. The polyacrylamide gel system used for the purification of tubulin was the discontinuous tris-glycine system of Laemmli (27) as modified for slab gels by Studier (28; see also 54). For preparative electrophoresis in gels 2 mm thick, 135 mm long, and 215 mm wide, a maximum of 400 μg of tubulin was applied without appreciable loss in the resolution. The thickness of the gel could be increased at least up to 4 mm to bring the loading capacity of the tubulin band to approximately 1 mg (see Fig. 1). The protein was dissolved at a concentration of 1–2 mg/ml in SDS sample buffer (0.1 M dithiothreitol, 2% SDS, 0.08 M Tris-HCl [pH 6.8], 15% glycerol and bromphenol blue) by placing the mixture for 3–4 min in a boiling water bath. The sample was layered on top of the upper gel and electrophoresis was carried out at 25–40 mA (125–150V) for 3–5 hr, (6–10 hr for slabs 4 mm thick). After electrophoresis, the gels were stained for 10–20 min in 0.25% Coomassie brilliant blue (CBB), 50% methanol, and 7.5% acetic

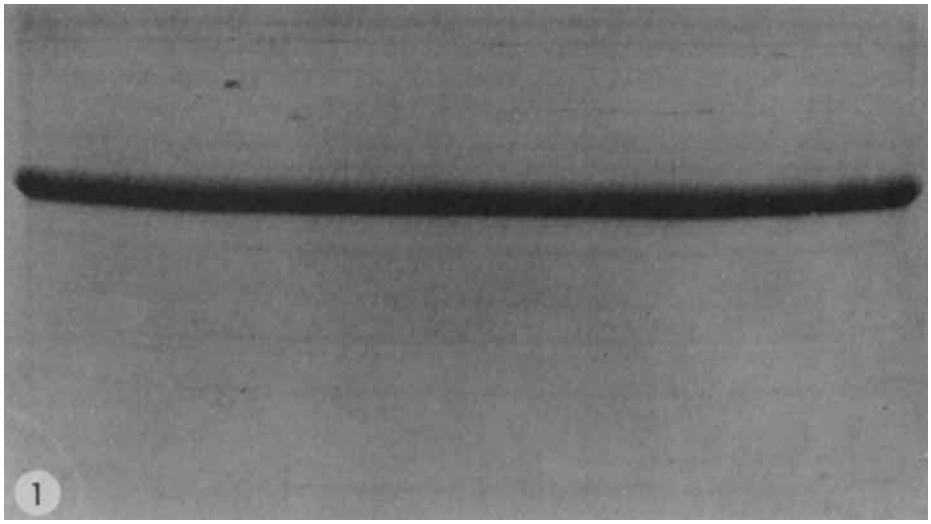


Fig. 1. Preparative electrophoresis of porcine brain tubulin. Aliquots (1 ml) of tubulin were electrophoresed on 12.5% polyacrylamide gels. The bands corresponding to tubulin (arrow) were removed from the rest of the gel for the preparation of tubulin as an antigen as described in detail in Methods.

acid, and destained in 20% methanol and 7.5% acetic acid on a rocking platform for 2–3 hr. After destaining of the slab gels, tubulin was removed from the rest of the gel with a sharp razor blade and eluted from the gel electrophoretically into a dialysis membrane as described by Allet et al. (30) for the elution of DNA fragments off gels, and by Anderson et al. (29) for the elution of proteins for subsequent peptide map analysis.

Elution columns were prepared from the lower 6 ml portion of a glass or plastic disposable 10 ml pipette (Fig. 2). About one-fourth of an inch was removed from the tip end of the pipettes to widen the hole and facilitate current flow. In all subsequent operations, care was taken to remove all air bubbles. The tip was plugged with a small amount of tissue paper and wet with 1–2 ml of SDS running buffer (per liter: 14.4 g glycine, 3 g Tris base, and 1 g SDS, pH 8.3). A knot was tied at one end of a small piece of dialysis membrane (1/4 in diameter) and cut so that there was about 2–2.5 in between the knot and the opening of the membrane. The membrane was filled with SDS running buffer, slipped onto the end of the pipette, and pushed up almost to the straight portion of the pipette; care was taken to insure it was secured tightly. The gel slices were cut into smaller pieces, neutralized briefly with running buffer, and packed in the elution tubes fairly tightly. The elution tubes were then filled with the upper gel mixture that is usually used as stacking gel (5% acrylamide, 0.13% N,N′-methylene bisacrylamide, 0.125 M Tris HCl [pH 6.8], and 0.1% SDS). After hardening of the gel, the top reservoir was filled with running buffer and the position of the tubes was adjusted so that the SDS-running buffer in the lower chamber was covering at least half of the dialysis membrane. The anode (+) was connected to the lower chamber and the cathode (–) to the upper chamber. Tubulin could be quantitatively eluted by 120–140 V applied for approximately 20–24 hr. The binding of CBB to proteins is pH-dependent and maximal at low pH. At the pH of the running buffer (pH 8.7) and under an electric field, the dye may dissociate from the protein and elute independently. Since the migration of the dye is faster than that of most

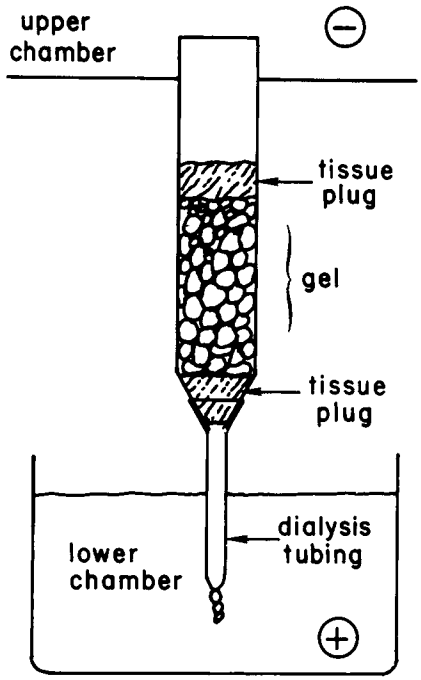


Fig. 2. Diagrammatic representation of the apparatus used to elute proteins after preparative electrophoresis in polyacrylamide SDS slab gels.

proteins, enough time should be allowed for the elution of the protein after the dye has accumulated in the lower part of the dialysis membrane. The recovery of tubulin from stained gels by this method was variable and ranged between 50–70% (as judged from the elution of radioactively labeled tubulin); the recovery of radioactively labeled actin by this method has also generally been 50–70%, while that of the heavy chain of myosin has been 40–50%. After elution, the upper part of the dialysis membrane was wet with SDS-running buffer and the tubing was removed from the column. The protein was concentrated after dialysis against two changes of 0.2 M NH_4HCO_3 + 0.05% SDS overnight (the dye does not dialyze out) by lyophilization and subsequent precipitation with acidified acetone (absolute acetone; 2–5 mM HCl). Tubulin (or actin) re-electrophoresed after elution and lyophilization had the same mobility on SDS-polyacrylamide gels as the original protein.

The lyophilized and acetone-precipitated protein was resuspended in a small amount of distilled water, and the flocculent protein solution, together with the remaining SDS and CBB, was emulsified with an equal volume of complete Freund's adjuvant. Emulsification with the adjuvant does not result in a complete mixture of the two solutions owing to the presence of SDS in the sample buffer. Complete emulsification can be achieved if the SDS-protein mixture is precipitated with $AlCl_3$ prior to emulsification (54). The immunization scheme used in white New Zealand rabbits was closely similar to that previously used for the generation of antibodies to α-actinin or tropomyosin (20, 25). Antibodies to tubulin could be already detected by indirect immunofluorescent examination of mitotic spindles (31, 55) 7–10 days after the second subcutaneous injection (500–600 μg of antigen; see also Ref. 19 for a similar response to actin purified in the above manner). The globulin fraction was purified from serum as described previously (20).

The properties of the antibodies generated against tubulin were quite similar to those generated against native tubulin partially cross-linked with glutaraldehyde prior to immunization, as described by Fuller et al. (31). The technique of preparative SDS gel electrophoresis has also been successfully used by Piperno and Luck in the preparation of antibodies against the β subunit of tubulin from Chlamydomonas flagella (32), and by Wiche and Cole (45) and Aubin et al. (46) for the preparation of antibodies to porcine brain tubulin.

Indirect Immunofluorescence

The indirect immunofluorescence technique used here was similar to that used previously (25) except that cells were dehydrated serially for 3 min in 24% ethanol, 10 min in 47.5% ethanol, and 3 min in 24% ethanol and returned to PBS for 3–5 min. The PBS contained 2 mM $MgCl_2$ but no Ca^{++} ions. If care is taken to avoid air-drying, the cell structure is considerably well preserved. It is not known if this method of fixation and dehydration preserves microtubules intact as seen by conventional electron microscopic fixation techniques. Using the indirect immunoperoxidase technique and the above method of fixation, antibodies to tubulin have been shown to interact with intact microtubules that extend for several microns into the cytoplasm. The use of ethanol or acetone up to 50% in the dehydration process is necessary for the diffusion of the antibody into the cytoplasm all the way up to the nuclear area and for its interaction with actin filaments or microtubules (unpublished observations). The material that is removed during the dehydration has not been systematically studied yet. Pictures were taken with a 100X Neofluar phase objective.

RESULTS

Immunofluorescent Consideration of Membrane Ruffling

The membrane ruffle of mammalian cells grown in tissue culture is a highly dynamic structure. Ruffles form by the upfolding of the membrane at the cell margin and exhibit continuous undulations, backfoldings, and eventually regression into the rest of the plasma membrane. They are frequently seen to fold downwards and attach to the underlying substratum or extend for several microns upward into the medium. Also, frequently they are seen to migrate backwards or inwards on the upper surface of the cell, thus producing "surface" ruffles. Such an example of side ruffling observed with the scanning electron microscope (SEM) in L6 cells is shown in Fig. 3. These cells also show a number of microvilli that are characteristic of the surface morphology of cells that exhibit extensive membrane ruffling. Since ruffling is such a highly dynamic and plastic manifestation of the plasma membrane, it is reasonable to assume that any of the cellular macromolecular structural components that are involved in the maintenance of the plasticity of the ruffling membrane should also be in a dynamic state of reorganization and reorientation. Since actin filaments and microtubules have been repeatedly inferred to mediate both motile and structural cytoplasmic functions, I examined the localization of these two antigens in ruffles using antibodies specific for either actin or tubulin. As Fig. 4 shows, actin appears to be one of the major constituents of the ruffling membrane matrix, whether the ruffling is taking place at the cell margin or on the surface of the cell. On the other hand Fig. 5 indicates that tubulin is not an antigenic component of the ruffling membrane but is restricted solely to the main cytoplasm of the L6 cell mostly in a diffuse form. These results demonstrate that actin is the principal structural component of ruffles

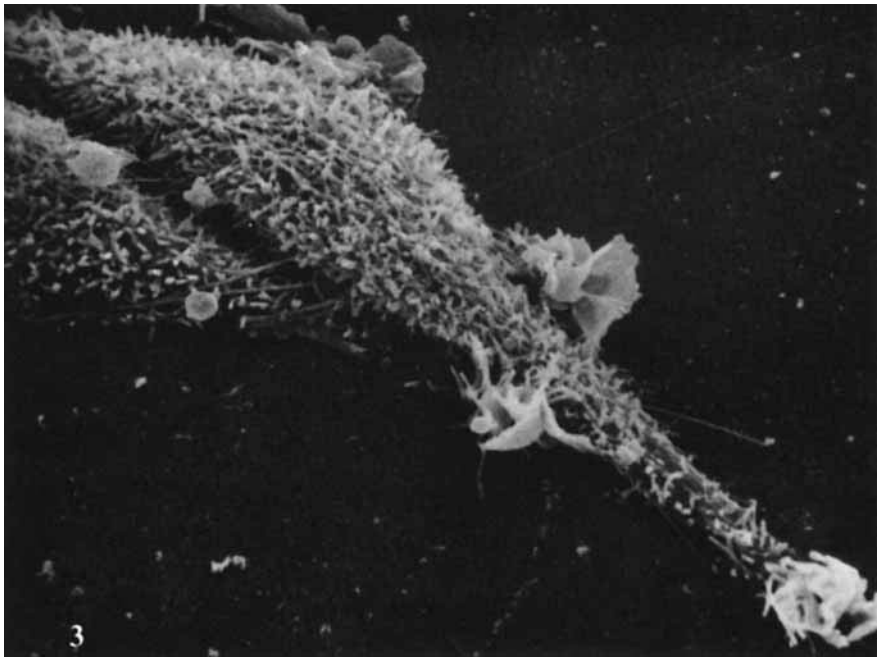
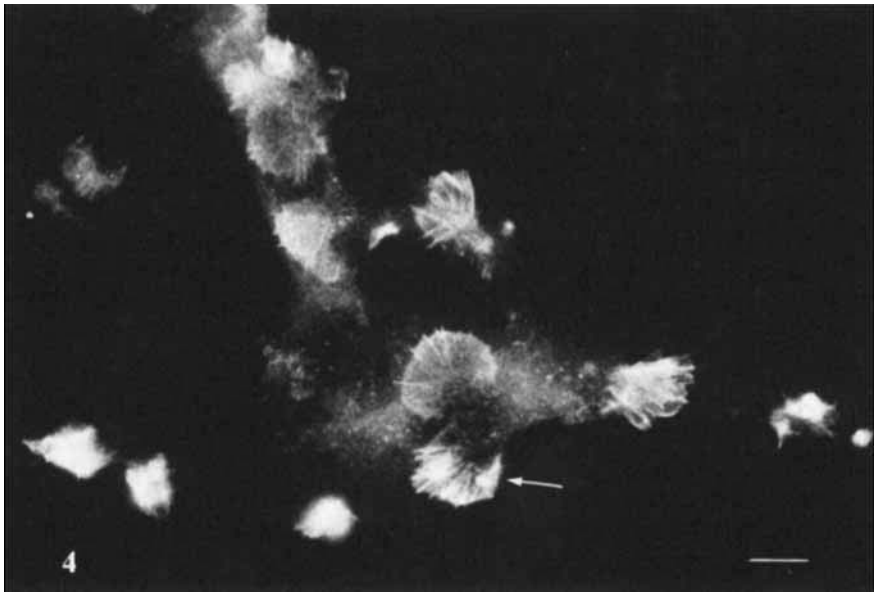
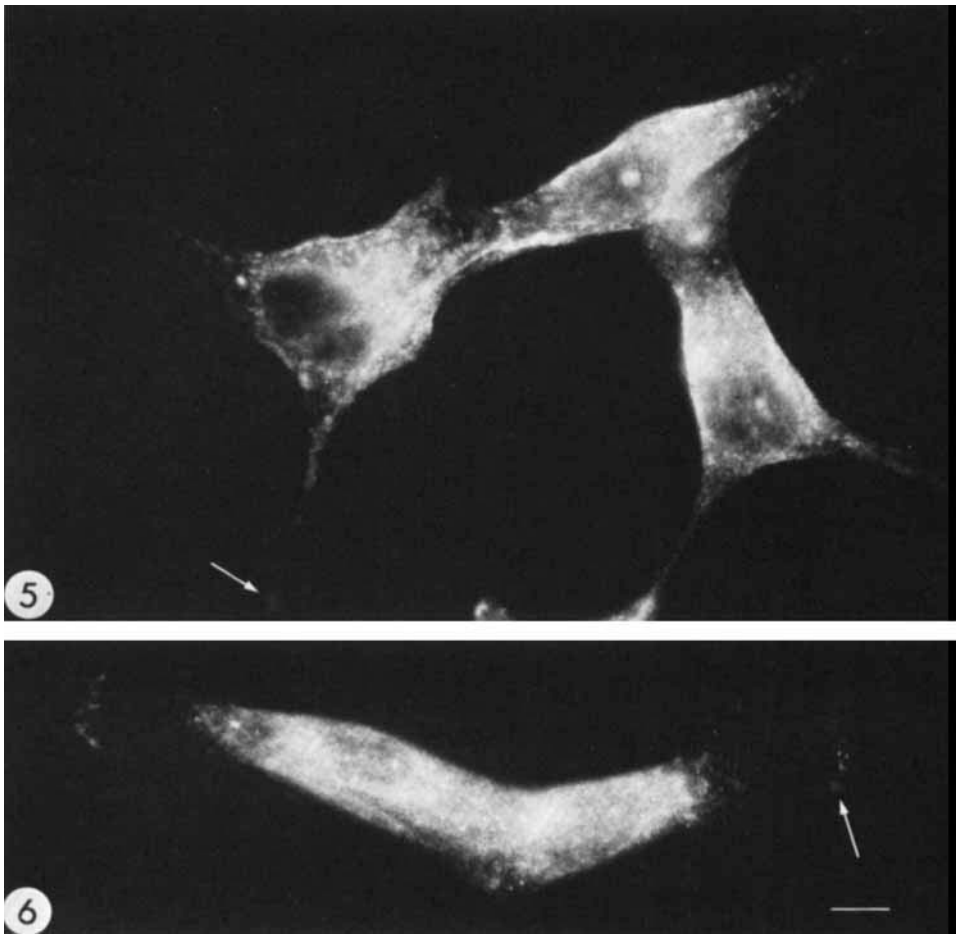


Fig. 3. Scanning electron micrograph of an L6 cell. The cells were seeded on carbon-coated 12 mm coverslips; 48 hr after plating the cells were fixed with 2% glutaraldehyde buffered with 50% Puck's saline G and 0.05 M cacodylate buffer (pH 7.4) and postfixed with 1% OsO_4 in 0.2 M cacodylate buffer. The cells were then dehydrated and critical-point dried as described by Porter et al. (44). The specimens were coated with carbon and gold (~ 200 Å) and viewed with a Cambridge S4 SEM (bar = 3.3 μm).



Figs. 4–6. Indirect immunofluorescence on L6 cells using antibodies to actin (Fig. 4), tubulin (Fig. 5), and tropomyosin (Fig. 6). The arrows indicate the presence of membrane ruffling. Note the absence of fluorescence from these areas in Figs. 5 and 6 using antibodies to tubulin and tropomyosin. Also note the filamentous structures visible in areas of membrane ruffling in Fig. 4 using the actin antibody (bar = 6.5 μm).



and that neither microtubules nor any other antigenically reactive form of tubulin participates in this membrane activity.

One of the most important cellular organelles that participates in the formation of membrane ruffles is the microspike. Wessells et al. (33) have thoroughly investigated the way microspikes participate in the formation of a specialized form of membrane ruffle, the growth cone found at the tip of elongating neuronal axons. These workers have shown that the microspikes that participate in the formation of the growth cone are highly dynamic structures that can transform from a highly fluid and plastic state to a rigid state. Microspikes are seen as an integral part of membrane ruffling and their outgrowth from the cell surface provides spatial guidelines between which membrane flows to form the ruffle. The ruffle obeys the same sort of movements that the microspikes do and it is expected that the underlying actin filaments will also undergo continuous changes in their state of organization or orientation to mediate these sorts of movements. These types of plastic movements are not characteristic of microspikes at membrane ruffles only, but appear to be the typical modes of movement of this organelle wherever they occur. As Albrecht-Buehler has shown, these sorts of microspike movements also take place in response to the sensory role that these organelles play in spreading cells or cells that come into contact with each other (34, 35). Figure 7 demonstrates further how surface microvilli

can extend into microspikes and fuse to form the spatial guidelines between which the membrane can flow and form the ruffle. These microspikes can also be seen to extend upwards above the membrane or extend for several microns and form temporary rigid contacts with the underlying substratum. These microspikes can also be seen to have local fluidifications in their membrane very similar to the fluidification of the membrane that has been observed by Albrecht-Buehler (35) at the tip of the microspikes of spreading mouse fibroblast cells. The mechanism by which surface microvilli extend into microspikes and fuse into the ruffling membrane is further illustrated in a stereo SEM picture in Fig. 10. Microvilli are seen to fuse and merge into the membrane ruffle, while microspikes extend from the ruffle into the surrounding environment. Indirect immunofluorescent examination of the ruffles with antibodies to actin reveals two types of fluorescence: a) an organized short filamentous form that frequently extends beyond the main body of the membrane ruffle (this short filamentous form of actin most likely represents the presence of actin filaments in microspikes); and b) a diffuse form that occupies the rest of the intrafilamentous space of the ruffle (see Figs. 4 and 9).

With the demonstration that actin filaments are the principal cellular structural components of the membrane ruffle, I turned my attention to an investigation of the possible regulatory mechanisms underlying such a process. Since tropomyosin has been previously shown to be intimately associated with fully assembled actin filament bundles in spread-out interphase cells (20), I examined the localization of tropomyosin in membrane ruffles using indirect immunofluorescence with antibodies specific for this protein. Figure 12 illustrates the unexpected observation that tropomyosin is antigenically absent from membrane ruffles under conditions where actin is present (compare the fluorescent images in Figs. 11 and 12). Two more such examples of the absence of tropomyosin from either the ruffles found at the edge of the cell or on the surface of the cell can be seen in Figs. 6 and 8. This molecule can be found, however, in a diffuse nonfilamentous form in the rest of the cytoplasm, indicating that tropomyosin is antigenically reactive in these cells. Similar observations on the absence of tropomyosin from membrane ruffles have also been made in a number of other cell types grown in tissue culture under a variety of circumstances where the ruffling activity is expressed (see also below). It appears therefore that tropomyosin is not an antigenic component of membrane ruffles. Under similar conditions α-actinin can be localized in membrane ruffles, suggesting that this molecule may mediate the attachment of at least some of the actin filaments to the undulating membrane of the ruffle, presumably in a manner similar to that by which it mediates the attachment of some of the actin filaments of the stress fibers to the plasma membrane elsewhere in the cell (Fig. 13) (5).

Immunofluorescent Studies on the Assembly of Actin Filaments and Microtubules During Cell Spreading

When cells are exposed to a proteolytic enzyme such as trypsin or to divalent cation chelators, such as EGTA or EDTA, they lose their high adhesiveness to the underlying substratum and they retract and assume a spherical phenotype. As we have previously indicated (25), this process of rounding up of the cell is followed by a concomitant and rapid disassembly of the actin filament bundles and their accessory proteins that are normally found associated with these filament bundles in fully spread-out cells. When these rounded-up cells are replated and allowed to attach to the underlying substratum, they slowly flatten out with a concomitant slow reorganization of their lost actin filament bundles. A similar process also takes place with microtubules. The highly elaborate net-

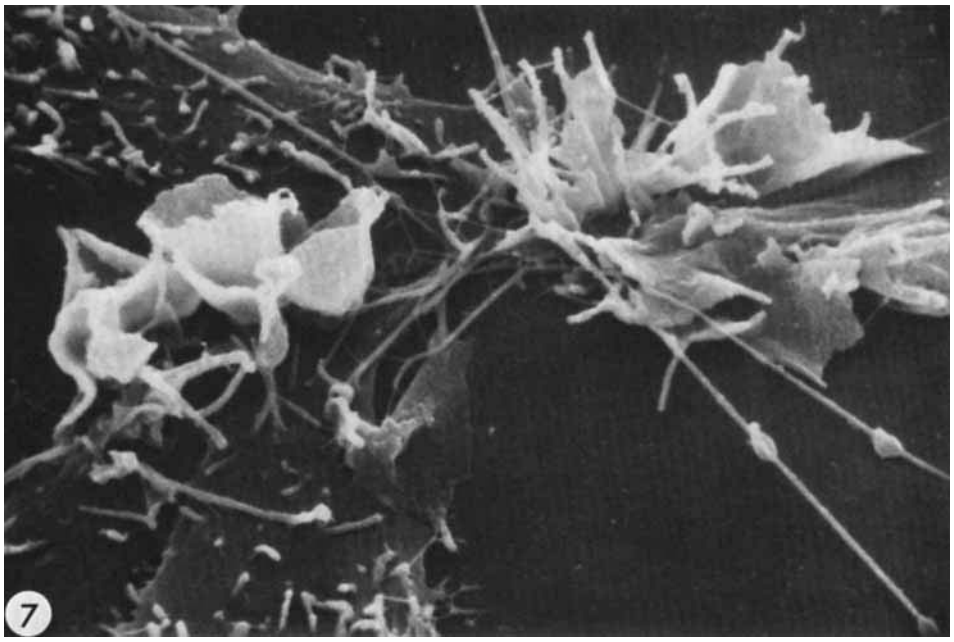
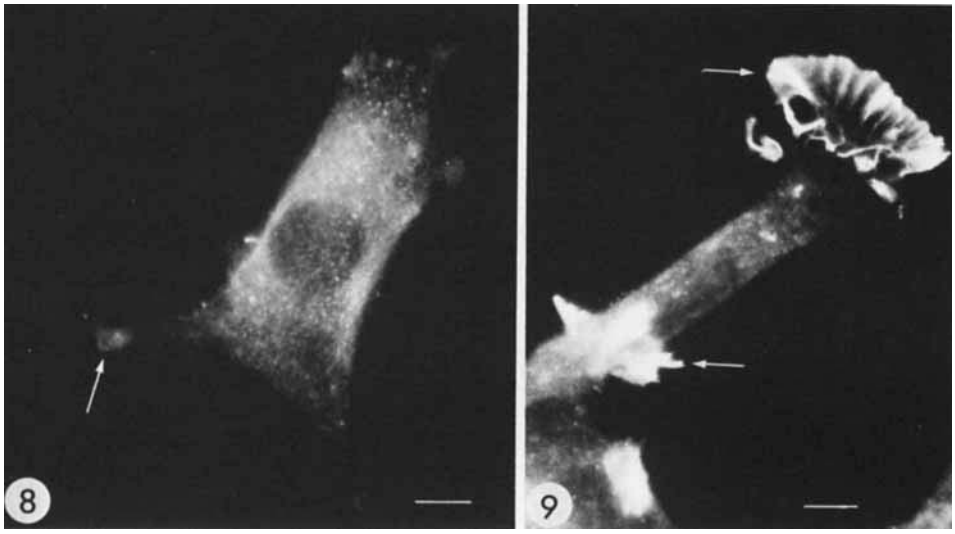


Fig. 7. Scanning electron micrograph of membrane ruffling in L6 cells. For details see legend for Fig. 3 (bar = 1.2 μm).

work of these thin filamentous structures that are normally seen in an interphase cell (55, 56) is disassembled when a cell rounds up, but it quickly reestablishes its lost pattern when the cells are allowed to spread out onto the underlying substratum. I have previously used the spreading of such cells onto a glass substratum as a model system to study the assembly of actin filaments as well as to study the role that α-actinin and tropomyosin might play in such a process (25). It was shown that in at least certain cell types actin filaments assemble through specific actin-α-actinin nucleation sites that are originally found in the area above the nucleus but are reiterated to cover the whole volume of the spreading cell and provide multiple sites of initiation and organization of assembly of the newly forming actin filaments. This process of spreading can also be used to study the assembly of microtubules or potentially any other tubulin-associated proteins that might be involved in this process. It can be similarly conjectured that multiple nucleation sites are also available in the growth of microtubules not only in the form of a centriole or a centrosphere in the perinuclear area (43, 56), but also in other forms in other areas of the cytoplasm or of the spreading membrane.

I have pursued further the assembly of actin filaments and microtubules during the first few hours of spreading of human lung cells (W138). During the first couple of hours after plating, spreading cells exhibit extensive membrane activity expressed in the form of expanding and rapidly regressing microvilli, microspikes, and blebs. Figure 14 shows that one of the principal structural components of these blebs is actin. The rest of the actin is localized in the form of a diffuse perinuclear fluorescence indicating that only a small percentage of the total cell actin participates in this activity. Since, as shown in Fig. 14 and previously (19), these active membrane phenomena are mediated by actin filaments, these filaments must be in a continuous state of reorganization and reorientation. In this



Figs. 8 and 9. Indirect immunofluorescence using L6 cells with antibodies to tropomyosin (Fig. 8) and actin (Fig. 9). The arrows indicate areas of membrane ruffling (bar= 6.5 μm).

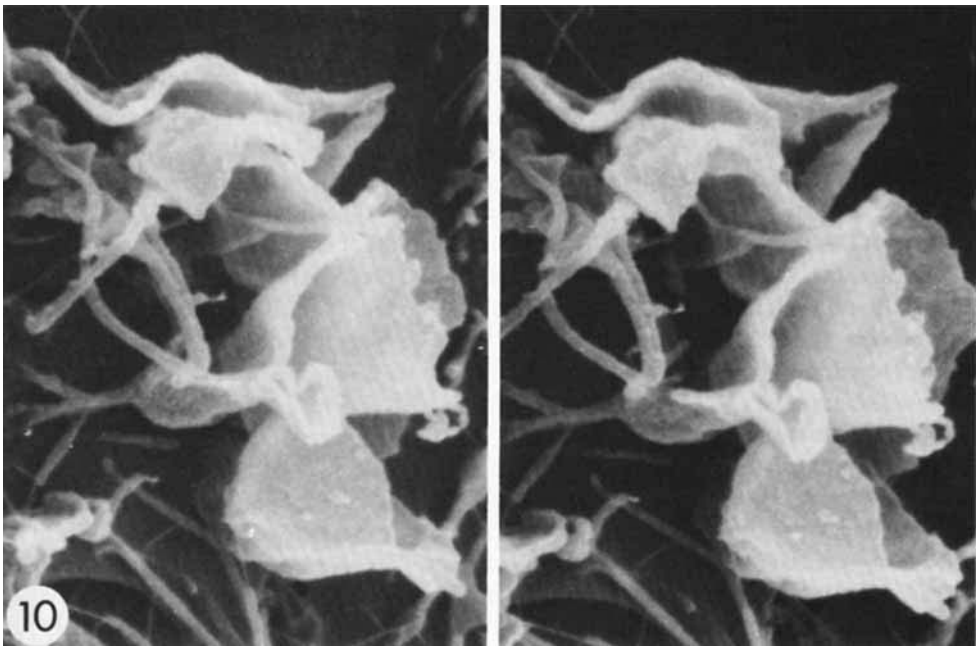
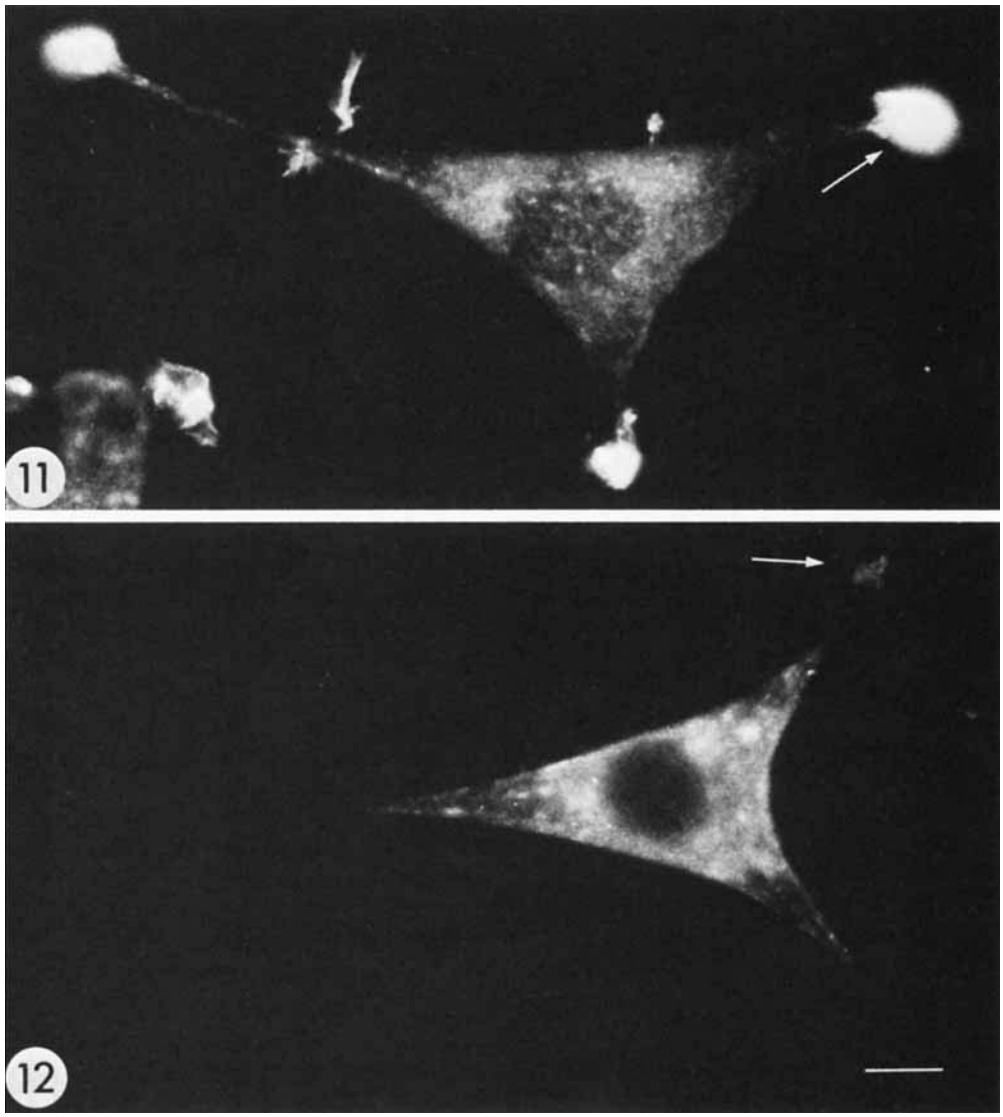
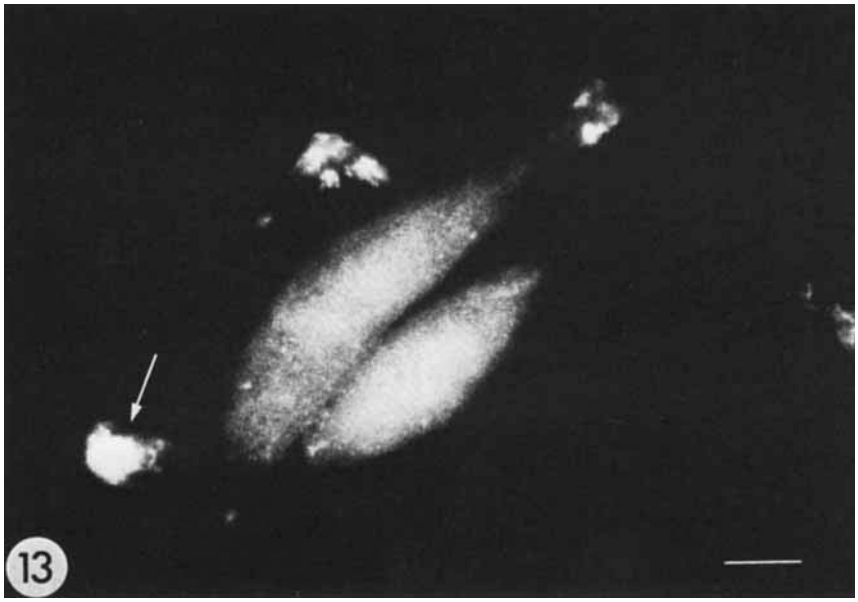


Fig. 10. Stereoscopic image of the ruffling membrane seen on the left part of Fig. 7. The left image was taken with a tilt of 40° while the right image with a tilt of 30° (× 9,000).

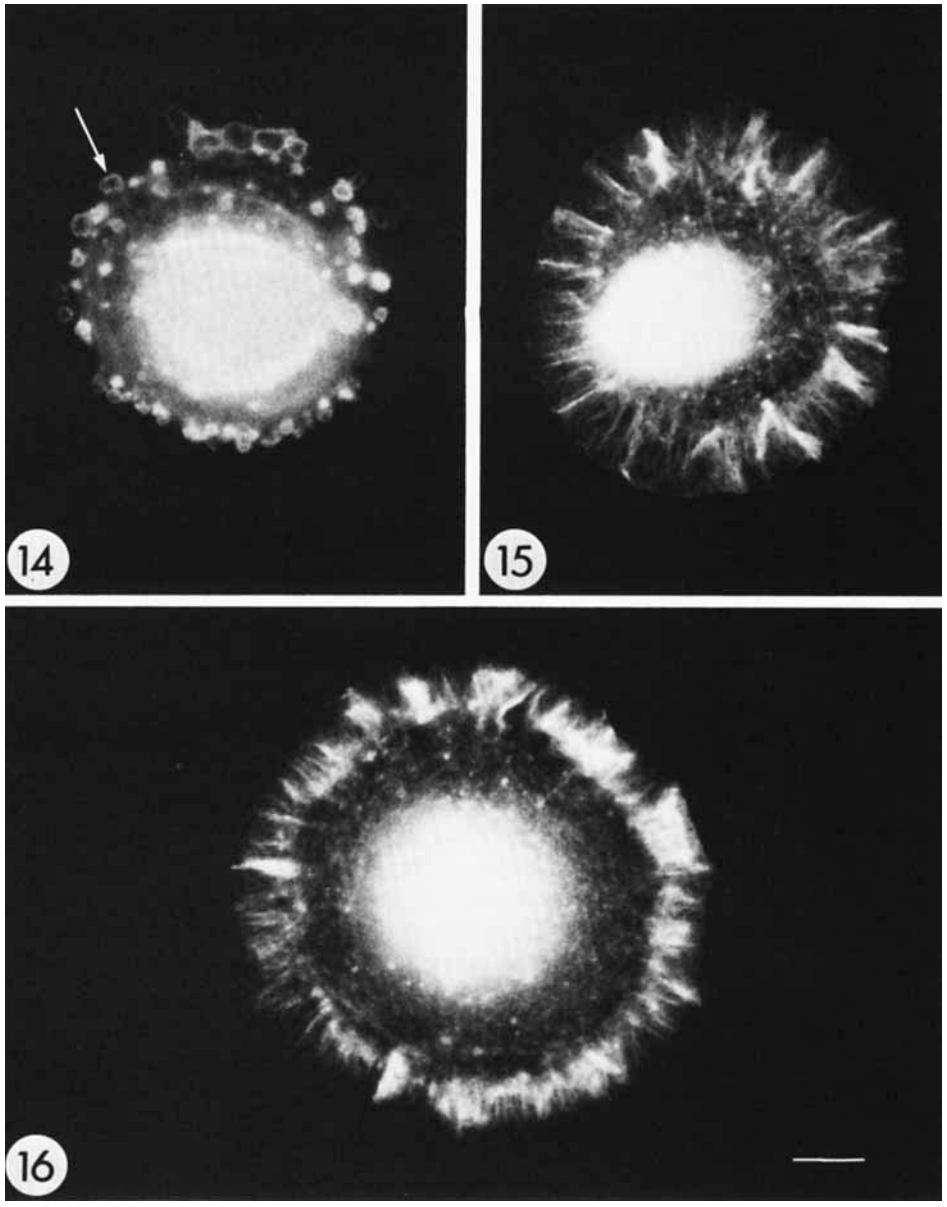
respect they differ from the highly aggregated state of these filaments in stress fibers. As the edge of the cell membrane continues to adhere and spread onto the substratum more actin appears to be mobilized to support this process. Figures 15 and 16 indicate that the actin filament bundles that are formed are short and positioned perpendicular to the spreading membrane. A number of wavy and bent actin filaments can also be seen in the background, once again indicating that these newly forming actin filament bundles are in a continous change of their state of aggregation in response to the continuous change of the outflowing membrane. Figure 16 also indicates that within the first hour of plating, the membrane at the cell margin begins to ruffle uniformly around the cell with a concomitant orientation of the newly forming actin filaments to support and mediate this activity. What determines this rather uniform length of the actin filament bundles at the cell periphery is presently unknown. However, Figs. 15 and 16 further indicate that these actin filaments leave a fluorescence-clear zone that differentiates the actin filaments engaged in the ruffling process from the as yet unmobilized diffuse actin fluorescence in the perinuclear region. When the cells are examined during this time with antibodies to tropomyosin, most if not all of the tropomyosin fluorescence is localized in a diffuse form in the perinuclear area (Figs. 17 and 18). It appears therefore that during these early stages of the dynamic assembly-disassembly of the actin filaments, the cell does not mobilize any tropomyosin to support any of the plastic motile membrane phenomena in which the actin filaments are involved. On the other hand antibodies to tubulin reveal a well-developed microtubule network (Figs. 19 and 20). At this stage of cell spreading (1 hr after plating) the human cells display two major localizations of microtubules. One population is seen to radiate from the nuclear area towards the edge of the spreading membrane, while a second population is seen localized in a circular fashion parallel to the spreading membrane (Fig. 20) (see also Ref. 56 for a similar distribution of microtubules in fully spread-out cells). Quite frequently the microtubules that radiate from the nuclear area are seen to bend and intermingle with the microtubules that run circularly, parallel to the spreading membrane. A closer examination of the microtubule patterns that are seen in Fig. 19 indicates that the ring of microtubules that run parallel to the spreading membrane is not as yet fully developed, but a number of radial microtubules are seen to bend and run for short distances parallel to the plasma membrane. In addition, however, the tubulin antibody reveals a punctate pattern of fluorescence, frequently along the path or as an extension of a microtubule. This punctate pattern is variable. Sometimes it permeates the whole cytoplasm and very few continuous tubules are visible, while at other times it is localized in certain areas of the spreading cell (Fig. 19) or it is absent and only continuous fluorescence is visible (Fig. 20). Within the first hour after plating α-actinin is found localized at the edge of the spreading membrane, presumably mediating the attachment of actin filaments at those membrane sites (Fig. 21). The α-actinin antibody also reveals a punctate pattern scattered in the cytoplasm. This pattern is distinct from that observed with the tubulin antibody and, as has been previously discussed (25), these fluorescent α-actinin sites may function as nucleation and organization sites for the growth of actin filaments. Figure 22 shows that the tropomyosin antibody reveals no fluorescence close to the edge of the spreading membrane or any punctate cytoplasmic fluorescence. This molecule is distributed in a diffuse perinuclear form unassociated with any newly forming actin-α-actinin filamentous complexes. Within the next 2 hr (2–3 hr after plating) the spreading cells begin to develop lamellae distributed symmetrically around the edge of the cell or to develop asymmetric processes. As Fig. 23 indicates, actin is not initially associated with the outflowing membrane, but becomes associated with it in the form of short filament bundles as the ruffling



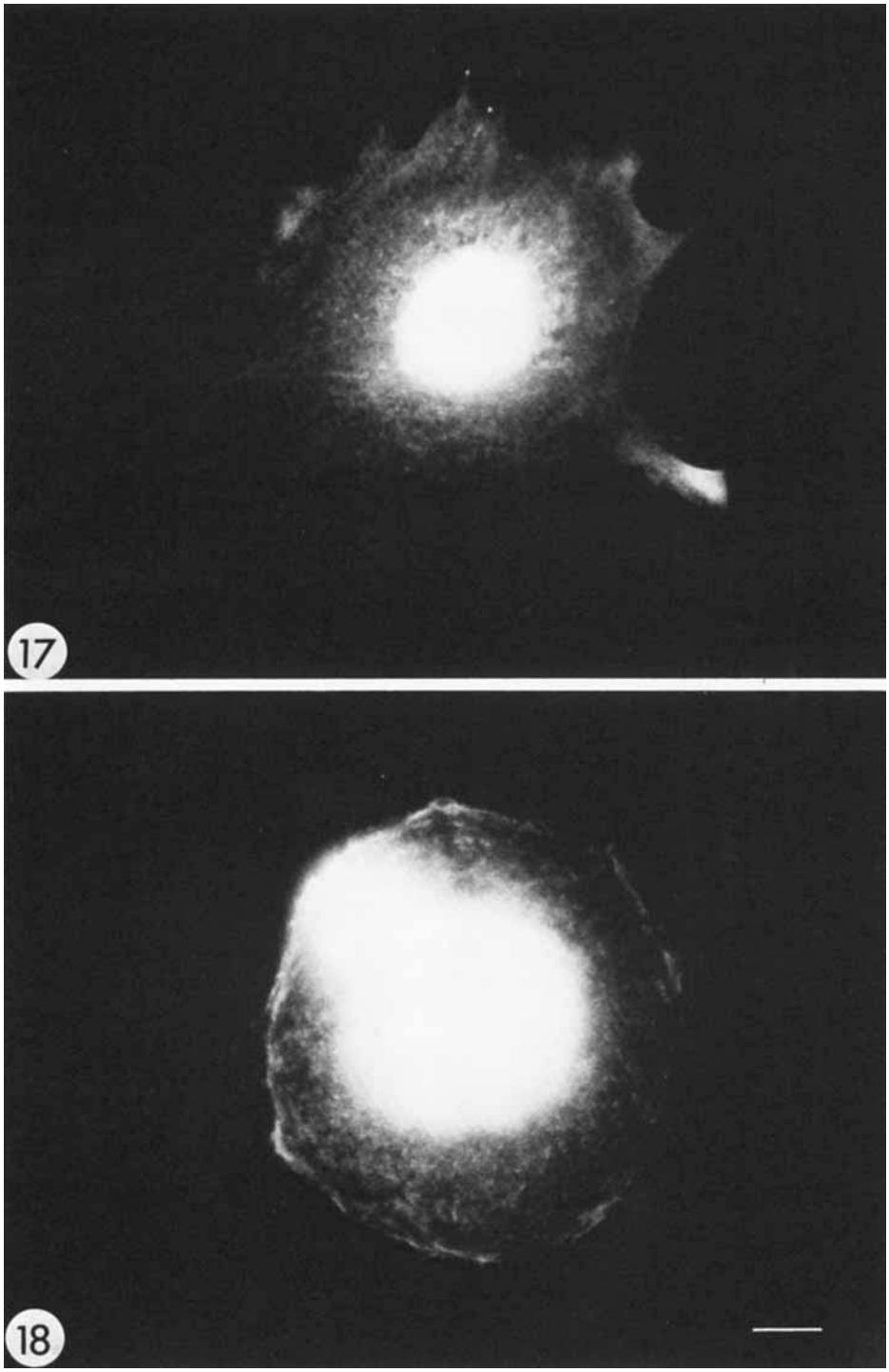
Figs. 11–13. Indirect immunofluorescence on L6 cells using antibodies to actin (Fig. 11), tropomyosin (Fig. 12), and α-actinin (Fig. 13). The arrows indicate areas of membrane ruffling. Note the absence of fluorescence from these areas in Fig. 12 using antibodies to tropomyosin (bar = 6.5 μm).



activity of the outflowing lamellae begins (compare Fig. 23 with Figs. 24 and 27). The rest of the cytoplasm reveals a number of homocentric circles of actin filament bundles as well as bundles of filaments radiating from the homocentric circles towards the edge of the spreading membrane (Figs. 23 and 24). However, both types of actin filaments, those associated with the ruffling membrane and those associated with the rest of the cytoplasm, leave a cytoplasmic area just behind the edge of the ruffling membrane that has only a very small number of actin filaments associated with it, giving the impression of a nonfluorescent dark cytoplasmic zone (compare Fig. 23, where the ruffling activity of the outflowing membrane has not yet begun, with Fig. 24, where it has). α-Actinin is distributed as previously in a punctate pattern throughout the cytoplasm, giving the appearance of homocentric punctate circles, comparable to those seen with the actin antibody (compare Fig. 29 with Figs. 23 and 24). In addition the α-actinin antibody reveals a number of fibers that radiate from the perinuclear area perpendicular to the edge of the spreading membrane (compare the α-actinin patterns in Fig. 29 with those of actin in Fig. 23). As noted previously (Fig. 20) the tubulin antibody reveals two populations of microtubules, those that radiate from the center of the cell toward the edge of the cell and those that run in a circular fashion parallel to the spreading membrane (compare the actin filament patterns in Fig. 24 and the α-actinin patterns in Fig. 29 with those of tubulin in Fig. 30). Quite often the microtubules that radiate toward the edge of the cell are seen to bend and intermingle with the microtubules that run parallel to the edge of the spreading membrane. Figure 28 indicates that despite the elaborate networks that actin filaments and α-actinin begin to develop, tropomyosin is still localized in the form of a diffuse perinuclear fluorescence and remains minimally associated with these newly developing fluorescent bundles. If, instead of forming a ruffling membrane symmetrically around itself, the cell



Figs. 14–22. Indirect immunofluorescence on a population of spreading human lung cells (W138) approximately 1 hr after plating using antibodies to actin (Figs. 14–16), tropomyosin (Figs. 17, 18, and 22), tubulin (Figs. 19 and 20) and α-actinin (Fig. 21). The arrow in Fig. 14 indicates areas of membrane blebbing. The arrow in Fig. 19 indicates the punctate fluorescence seen with the tubulin antibody (bars = 6.5 μm). Note the absence of any filamentous fluorescence in Figs. 17, 19, and 22 using the tropomyosin antibody. Also note in Fig. 21 the short filamentous form of α-actinin closely associated with edge of the spreading membrane as well as the punctate fluorescence elsewhere in the cytoplasm.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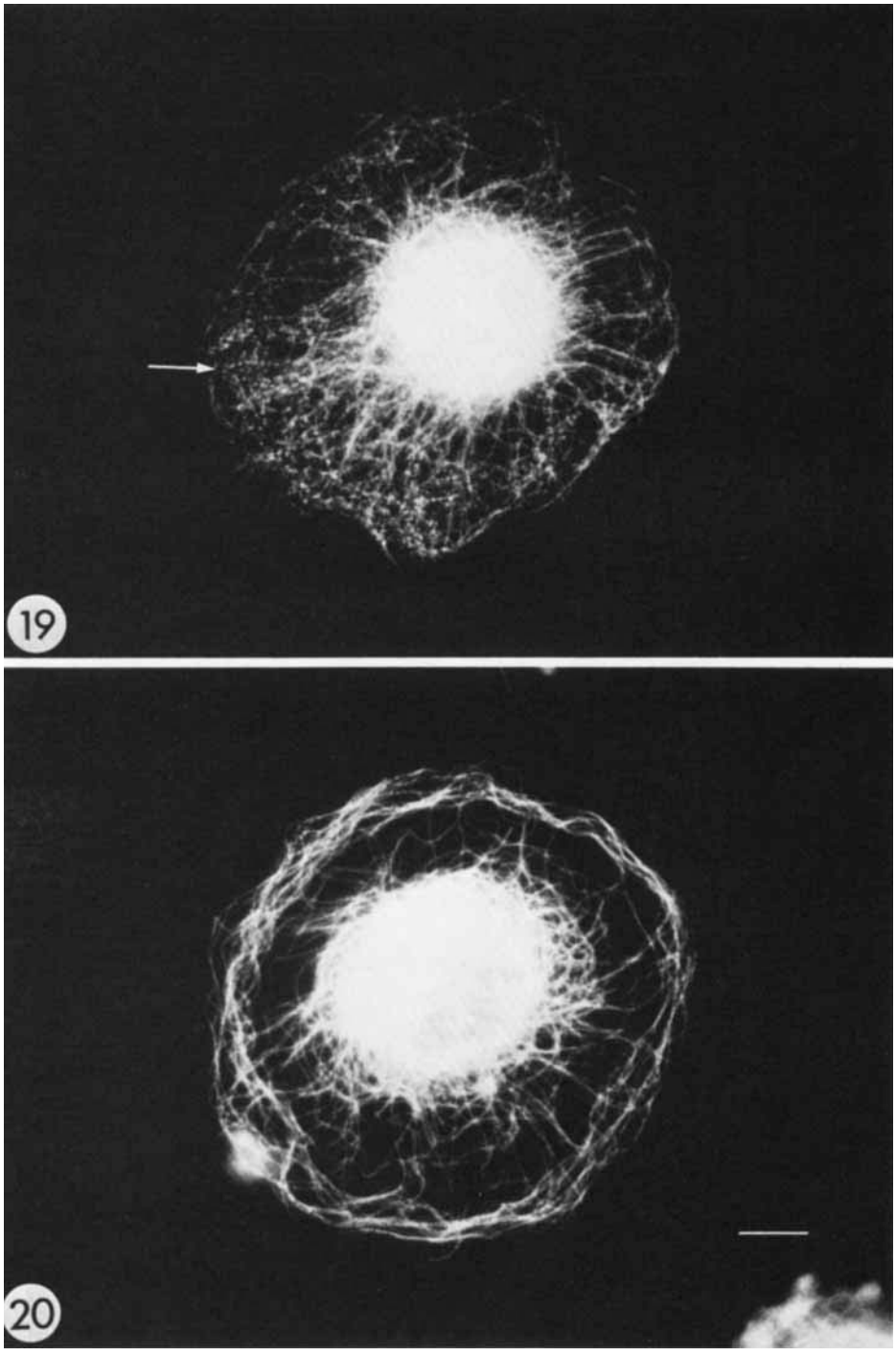
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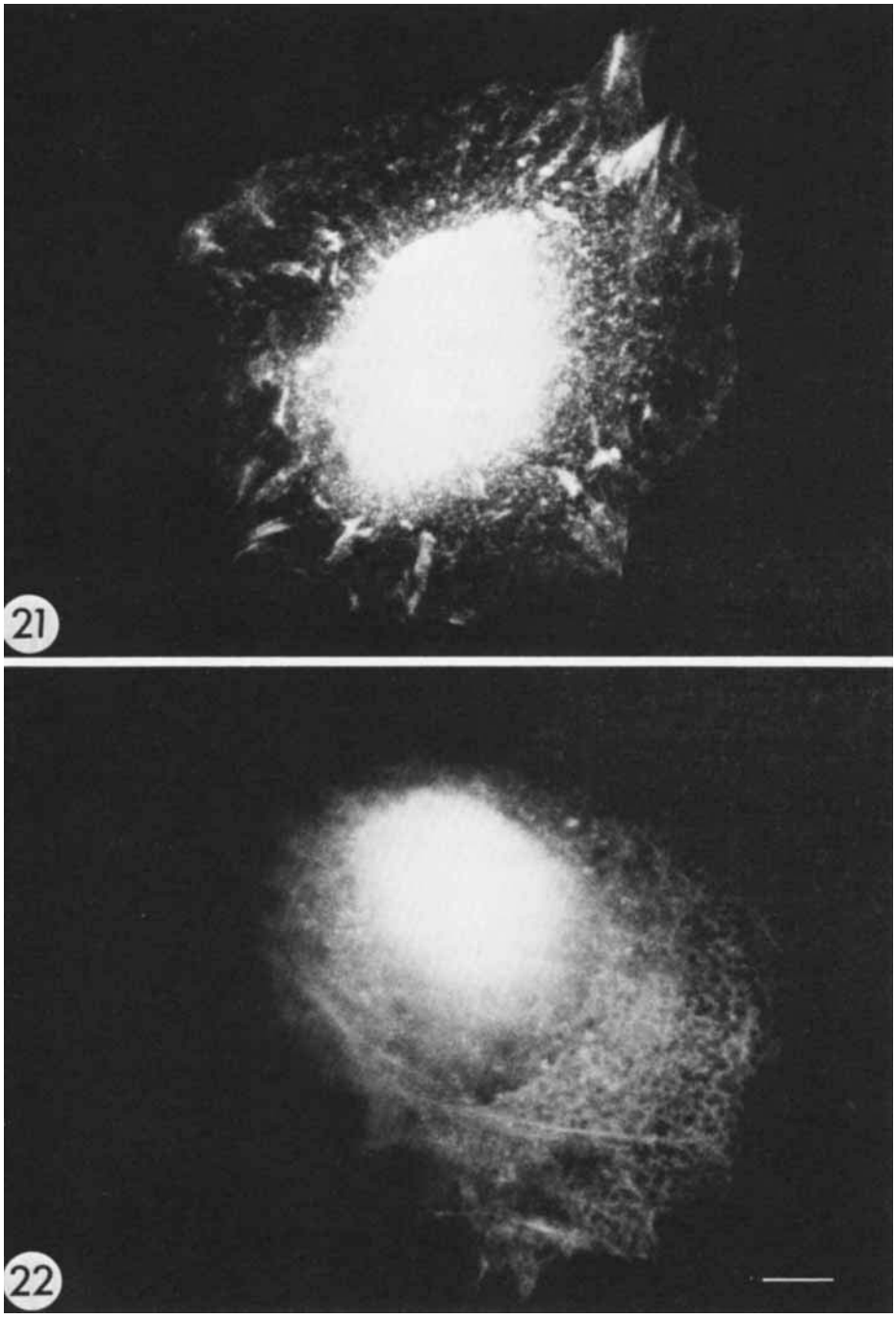
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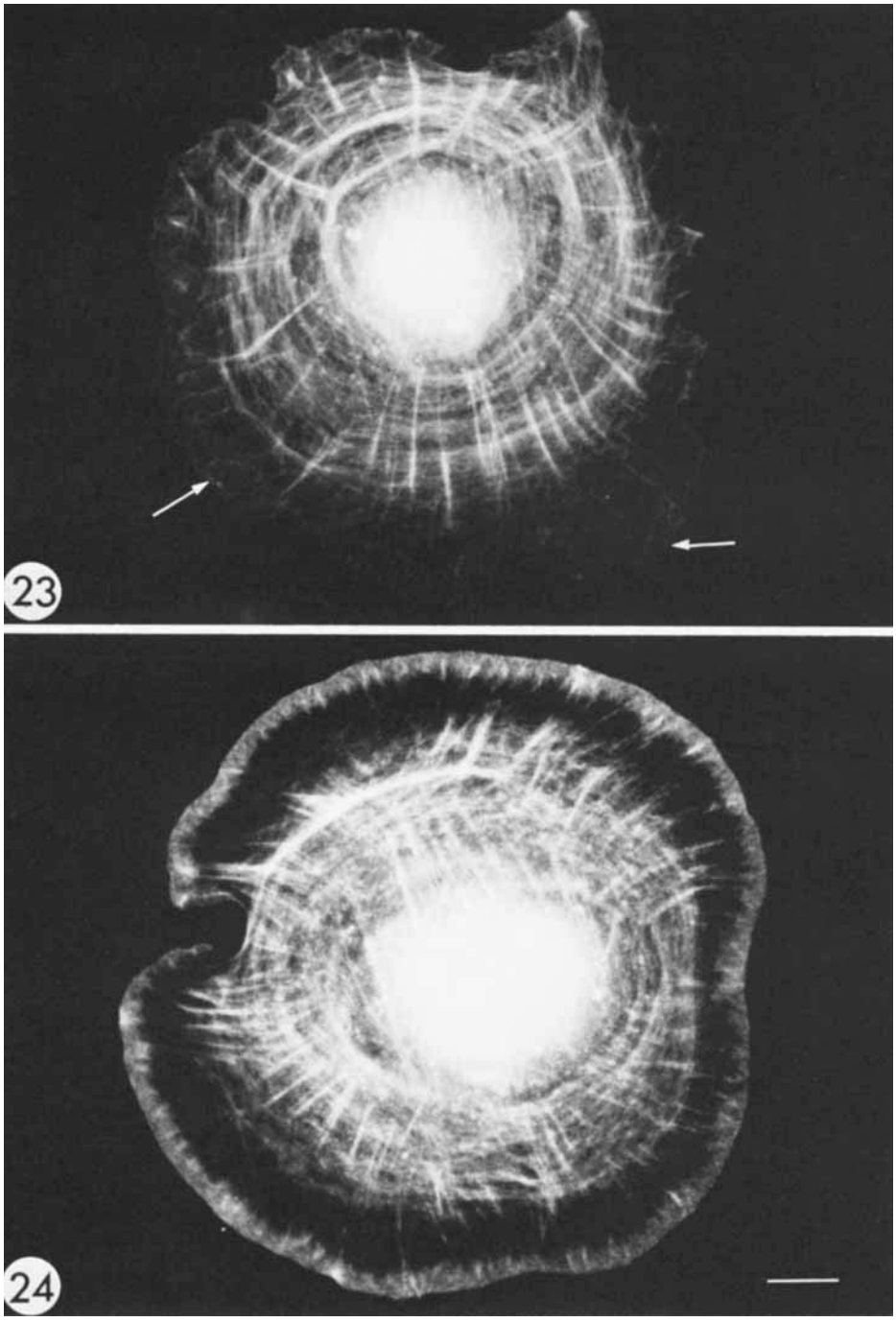
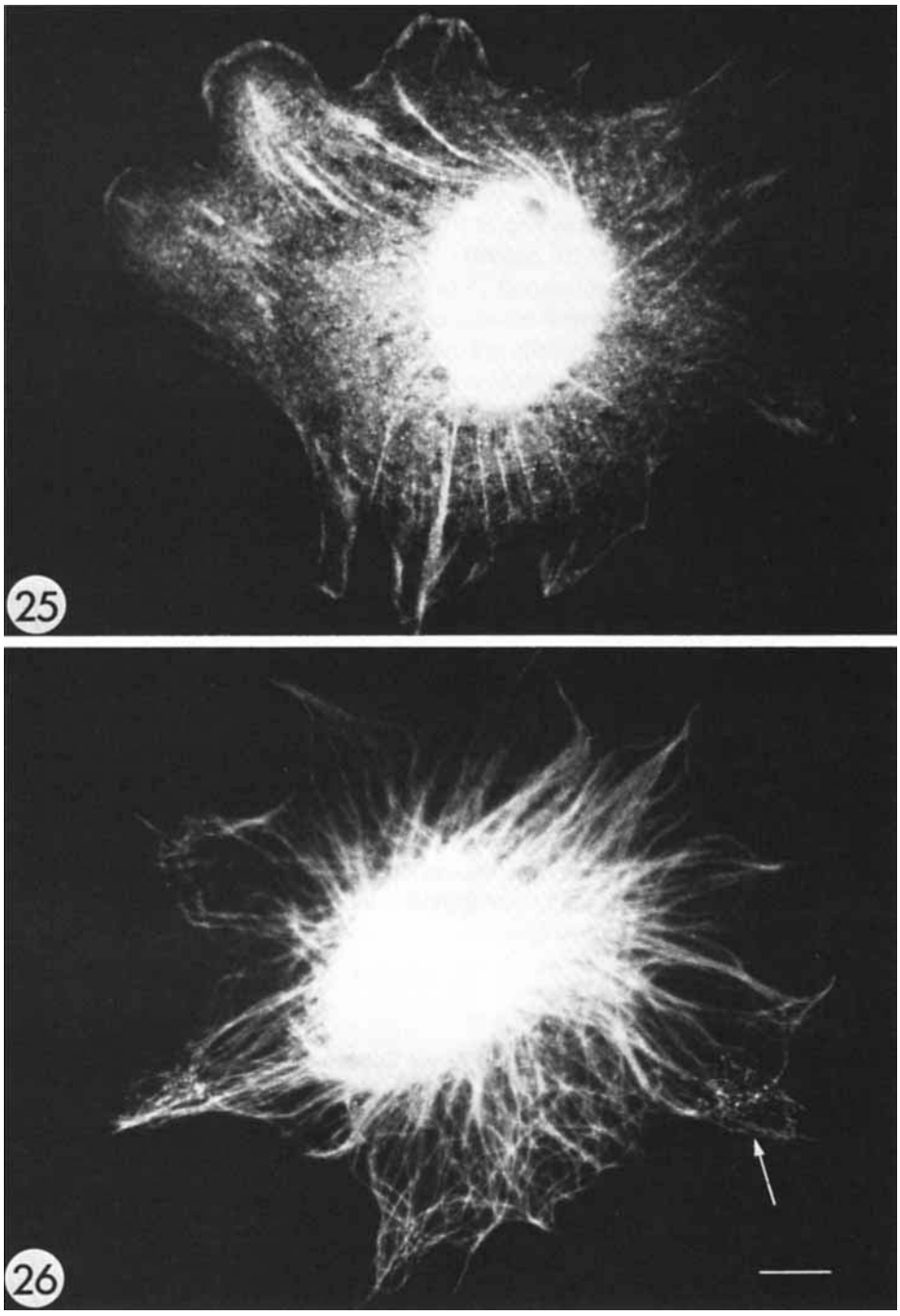


Figs. 23–26. Indirect immunofluorescence on the same population of spreading human lung cells as that used in Figs. 14–22 approximately 2 hr after plating using antibodies to actin (Figs. 23 and 24), α-actinin (Fig. 25), and tubulin (Fig. 26). The arrow in Fig. 23 indicates the leading edge of the



spreading membrane. Note the absence of fluorescence in this area using the actin antibody. Also note the fluorescence-dark area seen with the actin antibody behind the ruffling membrane in Fig. 24. The arrow in Fig. 26 indicates the punctate pattern seen in certain areas of the cell with the tubulin antibody (bar = 6.5 μm).

develops asymmetric processes, then both the actin filaments that radiate from the homocentric circles and the microtubules that radiate from the nuclear area are directed to polymerize toward these processes. In the case of actin this can be clearly depicted with the localization of α-actinin (Fig. 25) that is found intimately associated with this class of actin filaments. In the case of tubulin, the microtubules are seen to radiate from the nuclear area toward these processes without the presence of a well-developed network of microtubules that run parallel to the plasma membrane in these areas (Figs. 26 and 31). In some of these processes the microtubules appear to end in a punctate pattern similar to that noted previously (compare Figs. 26 and 19).

Within the next couple of hours (3–5 hr after plating) the cells begin to send out long lamellae that extend for several microns onto the substratum away from the main body of the cell. The main body of the cell cytoplasm is characterized by elaborate networks of actin filament bundles (stress fiber) as well as microtubules (Figs. 32, 33, and 36). The actin filament bundles appear to organize themselves in such a manner as to support the stretched phenotype of the cell (Fig. 32), but do not seem to extend into the main body of the lamellae (Figs. 32 and 33). The edges of the outflowing lamellae are characterized by intensive membrane ruffling, and actin filaments become intimately associated with this active process in a configuration that is quite distinct from that seen in the actin filaments of the stress fibers in the main body of the cell (Figs. 32 and 33). On the other hand the microtubules are seen to extend from the main body of the cell and occupy the main body of the lamellae and most likely become the structural supporting elements of these membrane extensions (Figs. 35 and 36). These microtubules end short of the membrane ruffling and in that area the tubulin antibody reveals distinct fluorescent foci (Figs. 35 and 36). However, neither these foci nor any form of tubulin appears to be associated with the very edge of the lamellae, where the active ruffling process is taking place. During this process tropomyosin is associated with neither the actin filaments found at the ruffling membrane nor the stress fibers in the main body of the cell (Fig. 34).

Within the next two hours, the cells are fully spread with a fully developed actin filament bundle network and a minimal amount of membrane ruffling. Tropomyosin can now be easily found associated with these fully developed stress fibers in a manner very similar to that previously described (20).

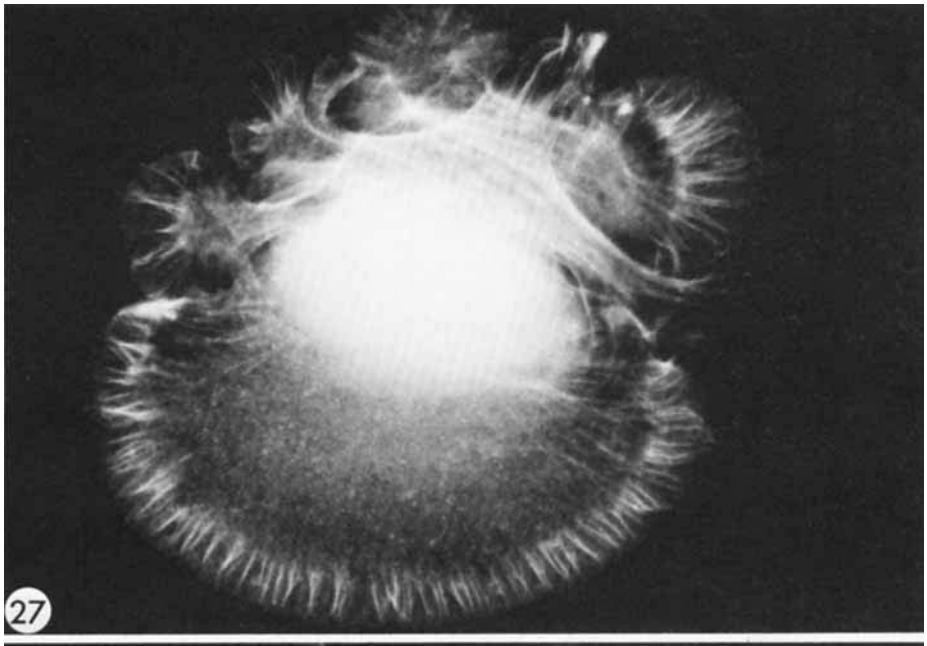
DISCUSSION

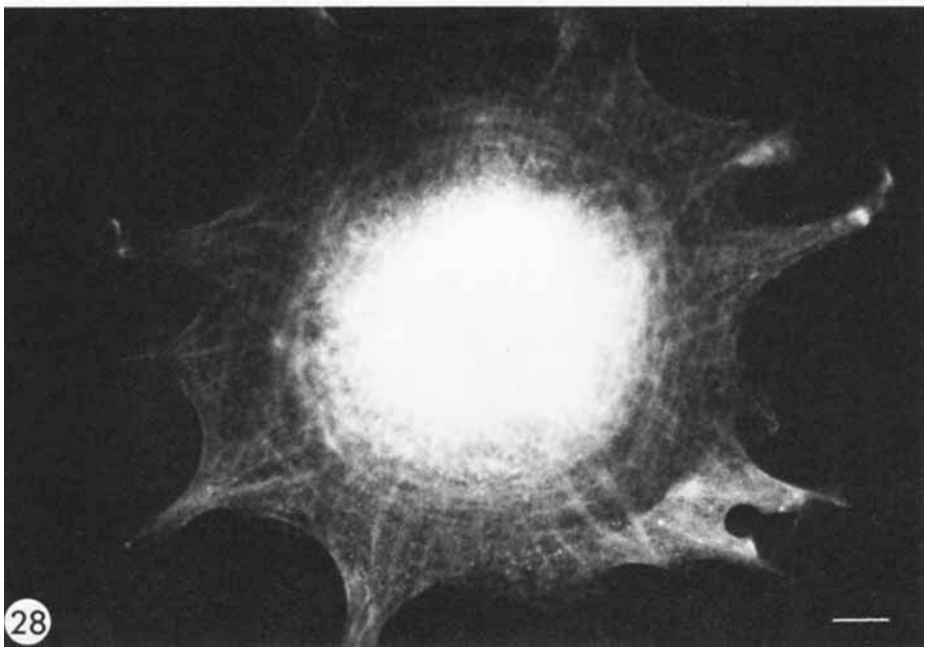
Two General Classes of Actin Filaments in Tissue Culture Cells: The Role of Tropomyosin

The results presented above in conjunction with some of the immunofluorescent results presented previously (24) indicate that tropomyosin is absent from the actin filaments found in the membrane ruffles as well as from the newly assembling actin filament bundles during cell spreading, but it is found associated with fully assembled actin filament bundles normally seen in spread-out cells.

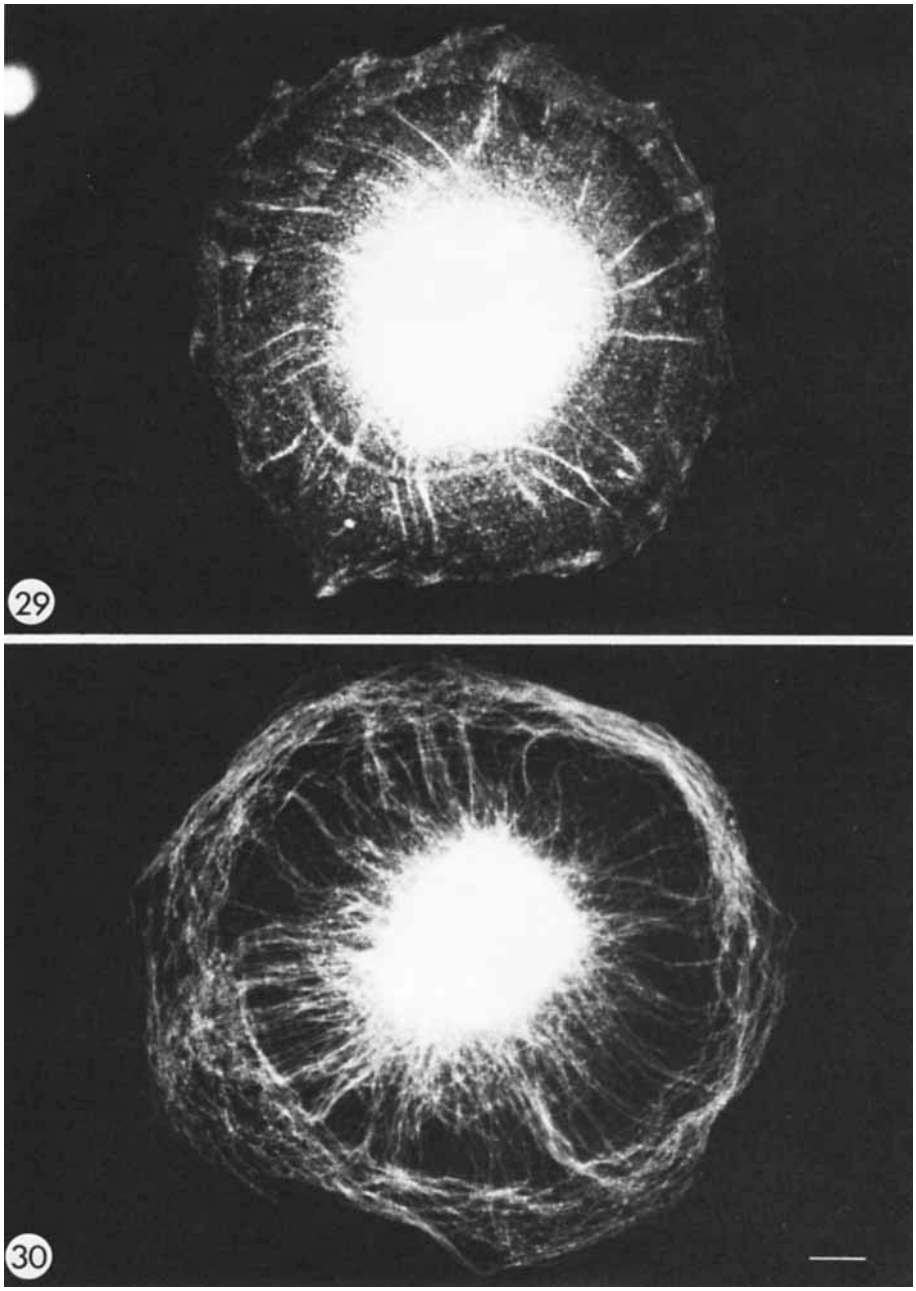
The lag that tropomyosin exhibits in its association with the newly forming actin filament bundles can be explained by any of the following possibilities: a) Tropomyosin is present on the newly forming filament bundles but it is antigenically masked and therefore the antibody cannot bind. This is a possibility that cannot be excluded in this sort of a negative result. It is a difficulty and a criticism that faces the absence of fluorescence from any specific place in the cell for any antigen. If it were the case, then the detection of tropomyosin in fully assembled actin filament bundles would result from a selective removal of this masking factor. Furthermore, such a factor would selectively inhibit the

binding of the tropomyosin antibody but not of the actin or the α-actinin antibody. In the following discussion this sort of antigenic masking is not considered as a primary possibility until future data point to the contrary. b) Tropomyosin is selectively degraded during cell rounding up, and the lag represents the time required for the molecule to be

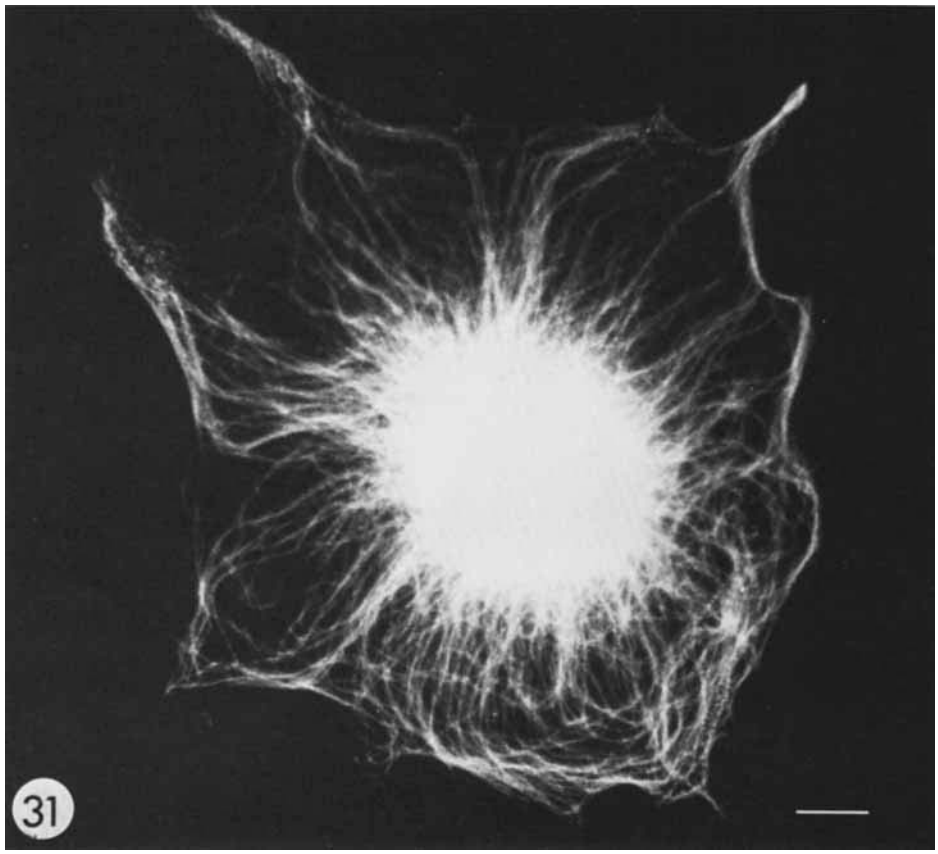




Figs. 27–31. Indirect immunofluorescence on the same population of spreading human lung cells as those used in the above figures approximately 3 hr after plating using antibodies to actin (Fig. 27), tropomyosin (Fig. 28), α-actinin (Fig. 29) and tubulin (Figs. 30 and 31). Note the distinctive punctate appearance of α-actinin in the cytoplasm of the cell depicted in Fig. 29 (bars = 6.5 μm).

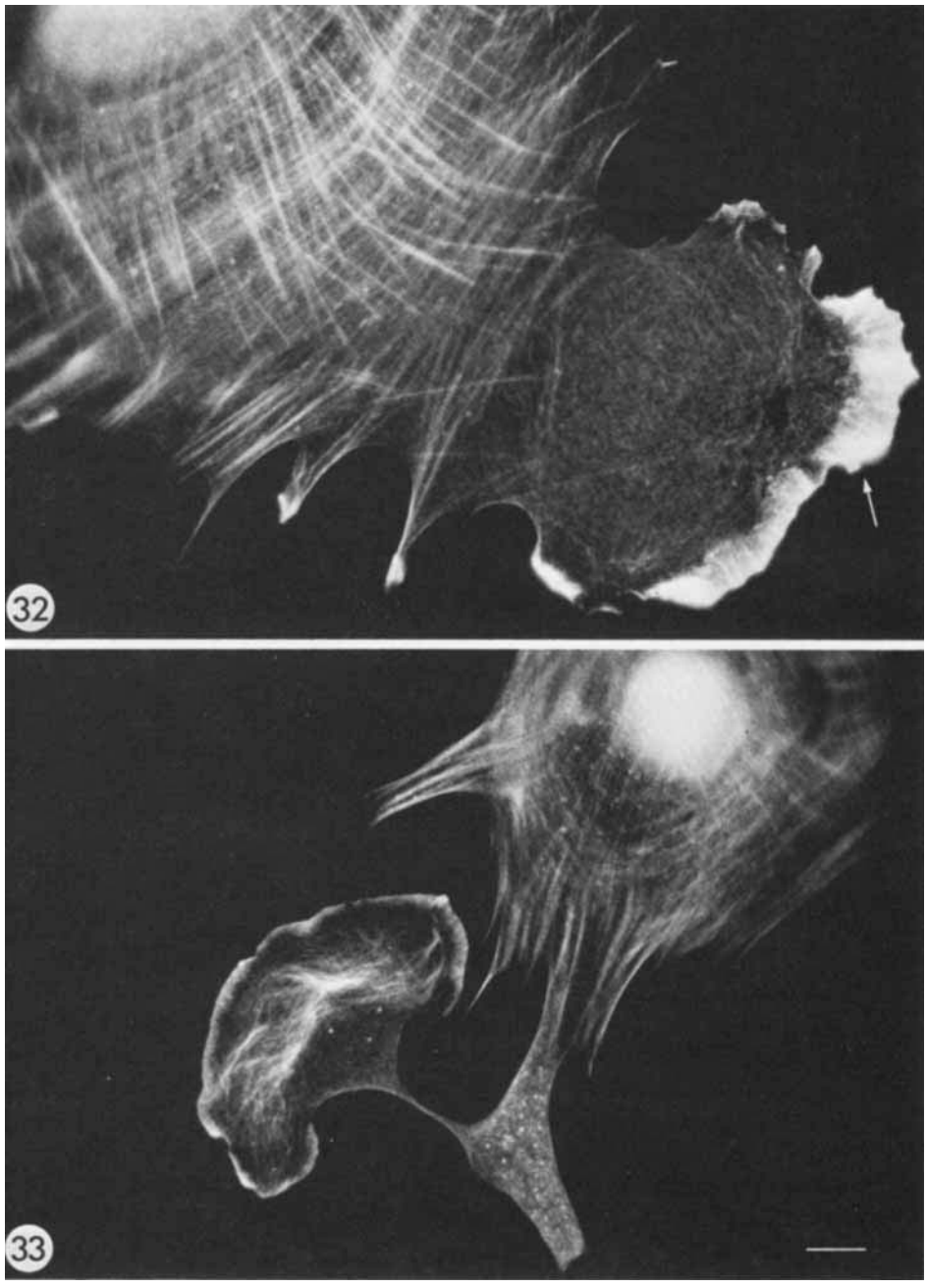


resynthesized before it exists in sufficient amounts to bind to the newly forming actin filament bundles. For reasons discussed in the Introduction this is most likely not the case. c) Newly forming actin filament bundles or actin filaments found in membrane ruffles contain a molecule bound to them that inhibits the binding of tropomyosin. In this case this inhibitor is removed from these filament bundles during the last stages of cell spreading by a selective process to allow the binding of tropomyosin. d) The fourth possibility is

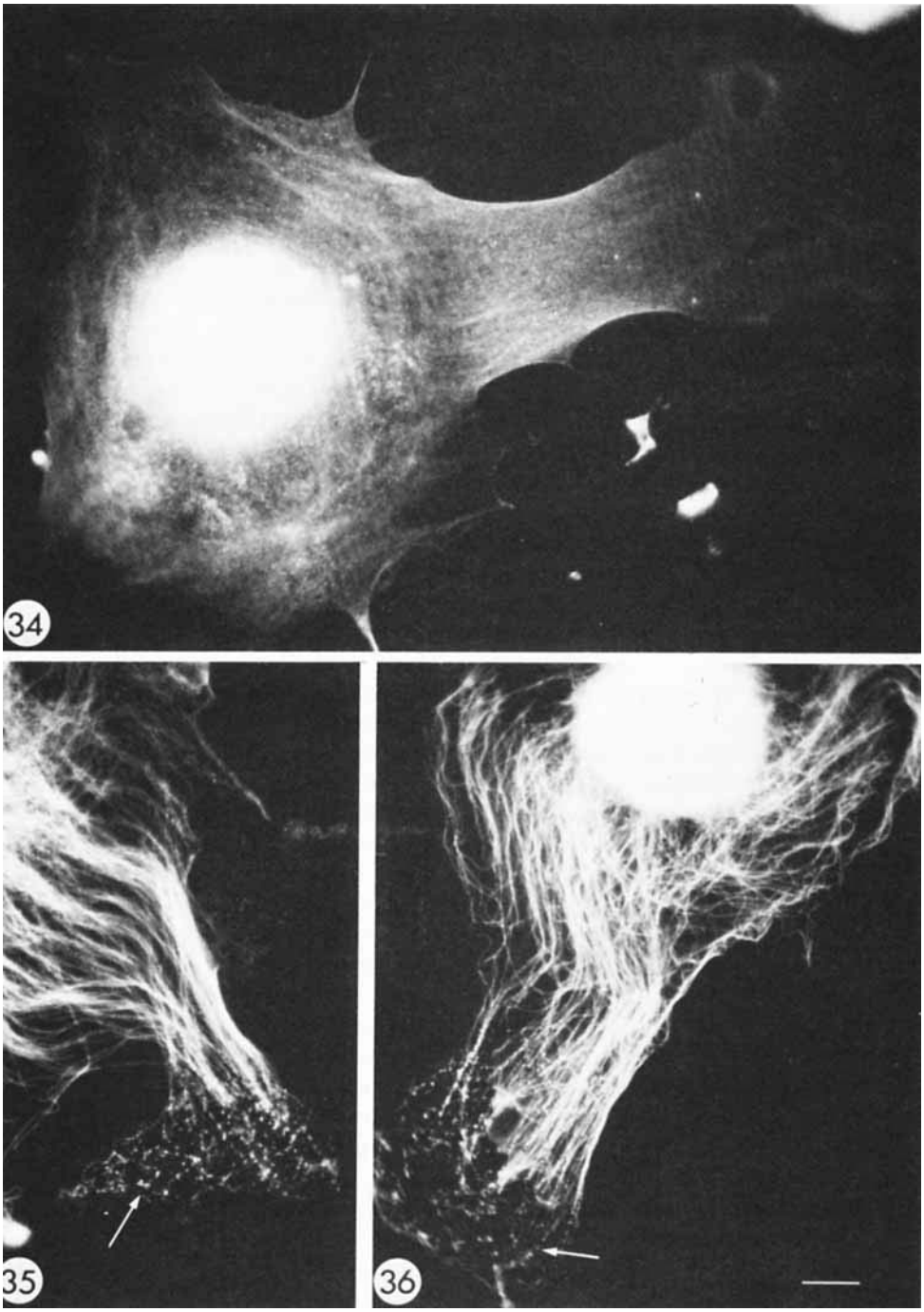


that fully assembled actin filament bundles are aggregates of two types of actin filaments: i) one class of actin filaments that has a molecule bound to it that excludes the binding of tropomyosin, and ii) a class of actin filaments that has tropomyosin bound selectively on them but excludes the binding of the other molecule found on the other set of actin filaments. The lack of experimental evidence makes it difficult at present to decide between c) and d) above. However, for the reasons given below, I feel that the latter possibility may be more likely to explain not only the lag of the association of tropomyosin with the newly forming actin filament bundles but also its absence from membrane ruffles.

It has been recently demonstrated that certain classes of actin filaments contain tightly bound on them a protein component with a molecular weight higher than that of myosin. This high-molecular-weight actin-binding protein has been found closely associated with actin filaments in the cytoplasm of sea urchin embryos (36), the acrosomal reaction of sperm (48), the cytoplasm of macrophages (37, 38), and the cytoplasm of acanthamoebe (41). A protein with a similar molecular weight from smooth muscle has also been recently localized antigenically in indirect immunofluorescence along the actin filament bundles seen in interphase cells grown in tissue culture (39). Under certain circumstances actin filaments can undergo in vitro a process of reversible gelation that is dependent on the presence of this actin-binding protein (36, 37, 41) and that can contract in the presence of myosin. Similarly in the case of the acrosomal reaction of sperm, the activation of actin



Figs. 32–36. Indirect immunofluorescence on the same population of spreading human lung cells as those used in the above figures approximately 4.5 hr after plating using antibodies to actin (Figs. 32 and 33), tropomyosin (Fig. 34), and tubulin (Figs. 35 and 36). The arrows in Figs. 32 and 33 indicate an area of membrane ruffling. Note the absence of actin fluoresence in the area behind the ruffle. Also note the mesh of filaments visible with the actin antibody in the area of membrane ruffling in Fig. 33. The arrows in Figs. 35 and 36 indicate the punctate pattern that is visible with the tubulin antibody towards the edges of the outflowing lamellae. Note the absence of any filamentous fluorescence in Fig. 34 using the tropomyosin antibody (bars = 6.5 μm).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filament aggregation is dependent upon this actin-binding protein (48). However both processes are independent of the presence of tropomyosin. On the other hand the actin filaments found in the microvilli of the intestinal brush border have tropomyosin associated with them, but not the actin-binding protein (49). Recent in vitro experiments on the polymerization of actin have indicated that cytoplasmic extracts of cells grown in tissue culture may contain at least two general classes of actin filaments: one class that has the high-molecular-weight actin-binding protein associated with it and that has the ability to form a filamentous gel, and another class that has tropomyosin associated with it (unpublished observations).

Since both the actin-binding protein and tropomyosin can be found in the fully assembled actin filament bundles, and since in vitro these two proteins may exclude the binding of each other on the actin filaments, it is my contention that fully assembled actin filament bundles may be aggregates of at least two classes of actin filaments: those that bind the actin-binding protein, and those that bind tropomyosin. In view then of this discussion, the lag of the association of tropomyosin with the newly forming actin filament bundles can be explained as follows. Newly forming actin filament bundles are those of the class of actin filaments that bind a protein like the high-molecular-weight actin-binding protein but not tropomyosin. As spreading progresses, actin filaments that have the ability to bind tropomyosin aggregate around or intermingle with the newly formed actin filaments, and the binding of tropomyosin can now be detected in indirect immunofluorescence. The biochemical difference between these two general classes of actin is presently unknown. It has been recently demonstrated that cytoplasmic actin exists in at least three distinct isozymic forms, presumably coded for by distinct genes (57, 58). It is possible that one of these forms of actin may have a higher affinity for tropomyosin, while another form may have a higher affinity for the actin-binding protein. Further biochemical experimentation is needed to help us decide between explanations c) and d) above or if indeed these newly discovered multiple forms of cytoplasmic actin have different affinities for different regulatory molecules. The absence of tropomyosin from the early assembling actin filaments found in membrane ruffles indicates that this molecule is not directly involved in either the initiation of assembly of actin filament bundles or the regulation of motility of membrane ruffles. Therefore other molecules besides α-actinin (25) must be involved in the early events of the assembly of actin filaments or the regulation of movement of the actin filaments in membrane ruffles. Such candidates are the high-molecular-weight actin-binding protein and myosin or protein factors that can induce actin to form supramolecular aggregates (53). The ability of actin filaments to reversibly aggregate, gel, and contract in the presence of the actin-binding protein and myosin makes this class of actin filaments the prime candidate as the structural and functional filamentous complex of membrane ruffles. The ability of this class of actin filaments to form a rigid yet flexible and maleable filamentous structure may provide the ideal structural support for membrane ruffles. It is quite likely that the diffuse form of actin fluorescence seen in ruffles represents this class of actin filaments that is capable of undergoing subtle local reversible gelation-retraction effects in response to the plasticity of the movements of the ruffling membrane. These reversible gelation-retraction effects in conjunction with the presence of small filamentous bundles (Figs. 4, 9, 32, and 33) may allow the actin in the ruffles to function as an elastic matrix that supports and mediates this undulating activity of the membrane. Furthermore, the presence of α-actinin in these ruffles may provide a mechanism by which some actin filaments can anchor to the undulating membrane by a mechanism presumably quite similar to that by which this molecule mediates the interaction of actin filaments with the plasma membrane elsewhere in the cell.

During cell spreading, actin appears to be mobilized from a diffuse perinuclear form

to aggregate into a continuously increasing number of filament bundles as well as to associate with membrane ruffles. This diffuse actin fluorescence is characteristic of all cell types examined so far that are highly motile or that exhibit a rounded phenotype, such as cells that round up for mitosis, cells induced to round up with trypsin or EGTA and EDTA, or highly tumorigenic cells such as Krebs ascites cells that grow in the peritoneal cavity of the mouse. The spherical phenotype of the cell hinders, therefore, the visualization of any actin-containing filamentous structures, and it may be assumed that this diffuse fluorescence represents a depolymerized form of actin. Recent experiments have indicated that when such fully rounded-up cells are lysed gently with the detergent Triton X-100, they can be seen to contain numerous actin filamentous structures that react with the actin antibody, but give a diffuse fluorescent image due to the fact that these filaments exist in a highly mingled state. This mingled state does not resemble the highly ordered state of these filaments that is seen in cells that have adhered and spread onto a substratum. The cytoplasmic extracts of these rounded-up cells can be induced to form a viscous gel, and these actin filaments seem to be responsible for this gelled phenotype, very similarly to the way in which actin filaments are responsible for the gelled phenotype of cytoplasmic extracts of other cell types that have a rounded phenotype, such as sea urchin embryos (36) or motile macrophage cells (37). Furthermore, when these filaments are separated from the rest of the cytoplasm and analyzed on SDS gels, they appear to contain the same basic polypeptide components as those filaments isolated from gelled extracts of sea urchin embryos or macrophages, namely actin, myosin, and the high-molecular-weight actin-binding protein (unpublished observations).

Further analysis of the actin from such cytoplasmic extracts of tumorigenic cells that have a totally rounded phenotype indicates that the majority of the actin molecules recovered in these extracts exist in a filamentous state. It is likely, therefore, that such cells that exhibit a rounded phenotype contain in their cytoplasm numerous mingled actin filaments, some of which may be responsible for keeping the cytoplasm of rounded cells in a highly viscous state. The fact that these filaments exist in a highly mingled state rather than the highly ordered state seen in spread-out cells, in addition to the rounded phenotype of the cell, is responsible, I believe, for giving rise to the diffuse fluorescence normally seen in rounded-up or poorly spread-out cells.

When a cell, therefore, begins to spread onto a substratum, actin filaments are induced to convert from such a highly mingled phenotype to an ordered stretched phenotype. The cytoplasmic factors responsible for such a filamentous transformation of actin are unknown, but must exist. From the spreading experiments reported here and elsewhere (25), this transition is at least dependent on the increasing adhesion of the spreading cell to the substratum. Such a suggestion gains more credence from the electron microscopic observation that actin filaments undergo a transition from a filamentous meshwork configuration to an oriented configuration as trypsinized cells resettle and begin to spread onto the substratum (50). At the area of membrane-substrate contact, actin filaments are seen to organize into bundles and to originate from these areas of membrane-substrate contact. A similar event appears to happen when two cells collide or when two ruffles of two different cells touch each other. Electron-dense material appears at the areas of membrane contact and subsequently actin filaments begin to organize in bundles from these electron-dense plaques into the cytoplasm (51). In both these instances we have presented evidence that such areas contain α-actinin and actin (5) and that the two events may be equivalent. However, although such a capacity of the membrane to transform actin from a meshwork state to a filamentous state can be suggested morphologically and immunocytologically the biochemical basis for such an event is unknown. The recent demonstration that specific cytoplasmic protein factors are required for actin to form such supramolecular

filamentous forms (53) may prove to be of great biochemical importance in understanding the transition of actin filaments from a meshwork state in a rounded or highly motile cell to the supramolecular highly organized filamentous forms seen in fully spread-out, less motile cells.

Seen from a different point of view, what is the cellular role of tropomyosin? The immunofluorescent evidence presented above indicates that tropomyosin may differentiate between the actin filaments that are involved in highly plastic movements like those of membrane ruffles and microspikes, as well as the actin filaments of transiently formed filament bundles, from those that are found in fully assembled stress fibers; it binds preferentially to the latter and precludes its binding from the former. The presence of tropomyosin on the actin filaments of muscle even in the absence of troponin (22) indicates that this molecule may have another role in the structure of actin filaments in addition to its negative regulatory role in the activation of the myosin ATPase during muscle contraction. It is my contention that this role may be structural. Since tropomyosin is known to be an α-helical molecule in both muscle and nonmuscle cells (6, 7, 40), the extended configuration of this molecule might induce actin filaments to be rigid and straight, thus allowing very little freedom of bending of the actin double helix. I would like to suggest that in nonmuscle cells tropomyosin differentiates between highly "plastic" and dynamic forms of actin filaments and highly organized structural forms of actin. It binds preferentially to the latter to induce structural rigidity to these filaments as well as to induce actin filaments to maintain a stretched, highly organized state. Furthermore, its absence from areas where actin filaments are involved in dynamic motile processes may allow these filaments to have a more flexible configuration and be in a position to change rapidly their state of polymerization, organization, or orientation, and bend or change their length in response to the plastic modes of movement of these cellular processes. Thus tropomyosin is seen to play a role of a "negative modulator" of motility in nonmuscle cells. Its absence reflects the ability of actin filaments to be engaged in plastic and dynamic forms of movement, while its presence may rigidify and stabilize these filaments into highly organized lateral aggregates. The comparison of the localization of tropomyosin in membrane ruffles and in stress fibers exemplifies this point but does not exclude the possibility that both forms of actin filaments, with or without tropomyosin bound to them, can coexist as is probably the case within the stress fibers. Whether actin filaments that have tropomyosin bound to them are capable of being activated by the detachment of tropomyosin to be involved in highly plastic modes of movement is presently unknown. Even if the details of the above discussion may need modification with time and further knowledge of the structure of actin filaments in tissue culture cells, the immunofluorescent results reported above demonstrate that the cytoplasm of certain cell types grown in tissue culture contain at least two general classes of actin filaments: those that bind tropomyosin and those that do not. The absence of tropomyosin from certain motile phenomena where actin filaments are involved, such as membrane ruffles, indicates that these classes of actin filaments are regulated by cytoplasmic mechanisms distinct from that by which tropomyosin (and troponin) mediates contractility in skeletal muscle. These observations open the possibility that different types of actin filaments engaged in different cellular motile phenomena in tissue culture cells may be regulated by a host of coexisting regulatory mechanisms, some as yet undetermined.

As Wessells et al. have discussed (33), the state of actin in highly plastic cellular motile phenomena such as membrane ruffles is difficult to assess due to the possible artifacts that may be imposed on the structure of actin filaments by the conventional fixation

techniques used for electron microscopy. Pollard and co-workers (41, 42) have in fact shown that the absence of actin-binding proteins and in particular tropomyosin from actin filaments results in a deterioration or even destruction of actin filaments when these filaments are fixed with glutaraldehyde and subsequently treated with OsO_4. The immunofluorescent results reported above clearly indicate that tropomyosin is absent from membrane ruffles, and in light of the results reported by Pollard et al. (41, 42) it is highly likely that the structure of actin filaments seen after glutaraldehyde fixation and osmication in electron microscopic examination of membrane ruffles may be to a certain extent artifactual. On the other hand the mildness of fixation used in the indirect immunofluorescence technique, the rapidity of the technique, and the stabilization effect that the antibodies may be expected to have on actin filaments, make it likely that the distribution of actin filaments in membrane ruffles as seen by the immunofluorescence technique closely represents their state in a cell prior to fixation. The above discussion also holds true for the electron microscopic examination of any actin filaments that can be shown in immunofluorescence not to contain tropomyosin. Another such example is the newly assembling actin filaments in spreading cells. Since the majority of those filaments do not contain tropomyosin, their electron microscopic images may be to a certain extent artifactual.

Microtubule Structure in Spreading Cells

Microtubules have been traditionally identified (and defined) morphologically in electron microscope thin sections as filamentous structures with an average diameter of 250 Å (43). The use of antibodies to tubulin in indirect immunofluorescence provides a different operational definition for these structures that does not depend solely on the identification of a microtubule as a structure with a diameter of 250 Å, but as an antigen. In this respect the antibody can detect in indirect immunofluorescence antigenic structures that are not readily identifiable by conventional electron microscopic techniques. It is difficult to assess that the structures seen by the electron microscope and identified as microtubules are a subset of those structures defined by the antibody. The latter technique should certainly extend itself and identify microtubules at least as conventionally defined by the electron microscope after glutaraldehyde fixation. However, glutaraldehyde fixation prevents the antibodies from entering the cell even after extensive alcohol or acetone extraction of the cell and appears to inhibit the binding of the antibodies to the antigen (unpublished observation). Electron microscopic examination of the cells following the previously used technique of formaldehyde fixation, acetone extraction, and air-drying prior to the application of the antibodies (20) has shown that the cell ultrastructure is severely damaged and there is extensive microtubule breakdown despite the fact that stress fibers are left intact. The technique of formaldehyde fixation used here, followed by mild alcohol extraction of the cell without any air-drying, results in appreciable cell preservation of ultrastructure as well as considerable preservation of microtubules. Using conventional electron microscopic techniques, microtubules can be found in the cytoplasm of cells that have a diameter of approximately 250 Å and that run for several microns in the cell (unpublished observation). Yet despite the fact that they are preserved, it is not yet known if their length and distribution at the end of the immunofluorescence technique is exactly what should be expected by conventional glutaraldehyde fixation. Furthermore it is still not fully documented whether the indirect immunofluorescence technique can resolve single microtubules at the light microscope level. Thus it is still unknown whether the images of

microtubules reported above represent the images of single microtubules or microtubule aggregates, despite the fact that they appear so convincingly as the images of single microtubules. Studies in progress have been undertaken to elucidate this point.

In light of the above discussion, the punctate patterns that are seen with the tubulin antibody is difficult to interpret. The most attractive explanation is that they represent extranuclear tubule growth sites but they can equally as well result from a selective degradation of tubules at these sites. Such patterns have been observed in a number of cell types so far, but further work is currently in progress to possibly improve microtubule fixation and decide whether these structures represent tubule breakdown products or a novel in situ intermediate in tubulin assembly. However, since these punctate patterns appear only at specific cellular sites where tubulin assembly or disassembly might be expected to be an active process, it is my conjecture that they may represent meaningful structures and they may reflect novel in situ assembly intermediates or elongation sites for microtubules. This might seem especially plausible, in view of the continuous fluorescence that is seen along the other microtubules elsewhere in the cytoplasm. The existence of two populations of microtubules in spreading cells, i.e., those that radiate from the nuclear area towards the edge of the cell and those that run in bundles parallel to the edge of the spreading membrane, poses the problem whether these are two distinct biochemical forms of tubulin or are the same type of tubulin differentiated only morphologically. In either of these two cases it is intriguing to speculate how the tubules that are parallel to the plasma membrane grow. Do they have independent initiation sites close to the edge of the plasma membrane (possibly the punctate fluorescent pattern seen with the tubulin antibody) or do they form as a continuous extension of the tubules that radiate from the perinuclear area of the cell? Further work is currently in progress to investigate the in situ growth of microtubules in cells that are spreading after trypsin treatment and replating or after mitosis.

ACKNOWLEDGMENTS

I am grateful to Dr. K. R. Porter for the use of his laboratory facilities during the course of this work, and to Drs. K. R. Porter and J. R. McIntosh for their numerous valuable discussions. I thank Mr. John Meek and Mr. Robert McGrew for their help in tissue culture and scanning electron microscopy. I was supported by a postdoctoral fellowship from the Muscular Dystrophy Association of America and by a grant from MDAA to Dr. K. R. Porter.

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