ULTRASTRUCTURE OF MICROFILAMENT BUNDLES IN BABY HAMSTER KIDNEY (BHK-21) CELLS

The Use of Tannic Acid

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ABSTRACT

After standard glutaraldehyde-osmium tetroxide fixation procedures, the majority of microfilament bundles in BHK-21 cells exhibit relatively uniform electron density along their long axes. The inclusion of tannic acid in the glutaraldehyde fixation solution results in obvious electron density shifts along the majority of microfilament bundles. Striated patterns are frequently observed which consist of regularly spaced electron dense (D) and electron lucid (L) bands. A striated pattern is also observed along many BHK-21 stress fibers after processing for indirect immunofluorescence utilizing BHK-21 myosin antiserum. A direct correlation of these periodicities seen by the light and electron microscope techniques is impossible at the present time. However, comparative measurements indicate that the overall patterns seen in the immunofluorescence and electron microscope preparations are similar. The ultrastructural results provide an initial clue for the ultimate determination of the supramolecular organization of contractile proteins other than actin within the microfilament bundles of non-muscle cells.

KEY WORDS microfilament bundles · myosin · striations · tannic acid

"Stress fibers" or "tension striae" have been described in many types of cultured nonmuscle cells for more than 50 years (3, 12, 25, 42). They have been seen in living cells by bright-field, phase contrast, and Nomarski differential interference microscopy (3, 8, 12, 25). Stress fibers exhibit positive birefringence with respect to their long axes when examined by polarized light microscopy (8, 12, 16). This latter observation indicates that fibrous proteins are oriented with their long axes parallel to the long axes of stress fibers. Early electron micrographs of whole-mounted cultured cells also revealed stress fibers (1, 28), but it was not until the late 1960's and early 1970's that it became apparent that they consisted of submembranous bundles of 5- to 7.5-nm microfilaments (3, 8, 12). Submembranous bundles of microfilaments (mfb) have now been described in many types of cells, (3, 8, 10–14, 26, 27, 34, 41). Furthermore, it has been demonstrated that a major component of microfilaments is similar to muscle actin. Evidence in support of their actin-like nature includes their morphology, size, and ability to bind rabbit skeletal muscle heavy meromyosin (HMM) or heavy meromyosin subfragment-1 (HMM S-1) to form arrowhead complexes (9, 17, 32–33).

More recently, fluorescence microscope techniques including indirect immunofluorescence with actin antiserum (12, 24) and the use of fluorescein-labeled HMM or HMM S-1 (31, 32)

have confirmed that stress fibers contain actin. Other immunofluorescence studies indicate that stress fibers contain proteins antigenically similar to myosin (6, 40, 43), α-actinin (22, 23), tropomyosin (20–22), and filamin, which is a high molecular weight actin-binding protein (39). Actin and filamin antisera, as well as fluorescein-labeled HMM or HMM S-1, reveal stress fibers which fluoresce brightly along their entire lengths (12, 24, 31–32, 39). However, immunofluorescence procedures with myosin, tropomyosin, and α-actinin antisera frequently reveal stress fibers with alternating dark and fluorescent regions (6, 20–23, 40, 43). These different staining patterns suggest that muscle-like proteins are highly organized within stress fibers and may in fact form primitive sarcomere-like units. However, it remains to be proven that the arrangement of native proteins within stress fibers actually reflects the fluorescence images, or whether there are intermittent regions which, for some reason, prevent the binding of antibodies to their appropriate antigens. This latter possibility could easily produce a false impression of the periodic arrangement of some proteins.

A prerequisite to resolving these problems, as well as others which are inherent in the limited resolution of the light microscope techniques, is a thorough understanding of the ultrastructural organization of proteins within the mfb comprising stress fibers. This must be obtained ultimately by electron microscope methods. Mfb usually appear as simple parallel arrays of microfilaments embedded within and surrounded by varying amounts of amorphous electron-dense material (see, for example, reference 12). In a few cases, indications of possible periodic shifts in electron density have been reported. It has been suggested that these regions of increased electron density may correspond to skeletal muscle Z-lines (see, for example, reference 41).

In this study, we utilize a simple procedure to enhance the structural information within the majority of mfb in baby hamster kidney (BHK-21) cells. This involves the inclusion of tannic acid in standard glutaraldehyde fixation solutions. Tannic acid has been used to enhance contrast and reveal the subunit structure of microtubules (4, 37), microfilaments (19), muscle actin filaments (19), and, most recently, to enhance the appearance of arrowhead configurations on HMM-decorated microfilaments (2). Ultrastructural observations of BHK-21 cells after tannic acid treatment reveal that many microfilament bundles possess a rather remarkable substructure consisting of regularly-spaced electron-dense regions. The periodicities or patterns generated are reminiscent of the images obtained by immunofluorescence techniques. Therefore, attempts are made to compare the periodicities seen by electron microscopy with those seen by immunofluorescence microscopy with BHK-21 myosin antiserum.

MATERIALS AND METHODS

Cell Culture

BHK-21 cells (35) were grown at 37°C in BHK-21 medium (Grand Island Biological Company [GIBCO], Grand Island, N. Y.) containing 10% calf serum (GIBCO), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and 50 μg/ml of streptomycin and 50 U/ml of penicillin. Stock cultures were maintained in 60-mm plastic tissue culture dishes (Lux Scientific, Inc., Newbury Park, Calif.) in a humidified CO_2 incubator. Confluent cultures were split and replated, using 0.05% trypsin-EDTA solution (GIBCO) to remove the cells.

Electron Microscopy

Subconfluent cultures of BHK-21 cells were fixed in the 60-mm plastic culture dishes in which they were growing. Cells were fixed at room temperature either in 1% glutaraldehyde in phosphate-buffered saline (PBS; 6 mM Na^+-K^+ phosphate buffer, 171 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ at pH 6.8–7.0) or in this same solution containing 0.2% tannic acid (2) for 30–45 min. After either of these prefixation conditions, the cells on dishes were rinsed for 60 min at room temperature with 4 or 5 changes of PBS, postfixed in 1% osmium tetroxide in PBS for 1 h, and rinsed 2–3 times in H_2O for 1-min intervals. The cells were then soaked in 1% aqueous uranyl acetate for 45 min at room temperature, rinsed twice with water, dehydrated in a series of increasing concentrations of ethanol, and embedded in a thin layer of Epon-Araldite (38). After polymerization, the plastic containing the cells was removed as a disk from the dishes. Pieces of the disk were cut out and mounted on the ends of preopolymerized blocks of Epon-Araldite and sectioned with a diamond knife on an LKB Ultrotome IV (LKB Instruments, Inc., Rockville, Md.). Gold-silver sections were collected on uncoated 150-mesh copper grids and stained for 30 min in 3% aqueous uranyl acetate and then in lead citrate for 10 min (29). Thin sections were observed and photographed in a Philips 201C electron microscope.

Immunofluorescence

BHK-21 cells were prepared for indirect immunofluorescence with BHK-21 myosin antiserum after fixation or glycerination as described previously (43). All obser-

vations were made with either a Zeiss 63X apochromat (NA 1.4) oil immersion lens (Carl Zeiss, Inc., N. Y.) or a 100X planachromat (NA 1.25) oil immersion lens fitted to a Zeiss IIIRS epiilluminator containing a narrow band FITC filter system. All fluorescence photomicrographs were taken on a Zeiss Photomicroscope III.

RESULTS

Ultrastructure of MFB After Fixation Without Tannic Acid

The majority of mfb seen in thin sections of flat-embedded BHK-21 cells are localized in the cortical cytoplasm adjacent to the cell surface in contact with the substrate (12, 14). With glutaraldehyde fixation without tannic acid, most of the mfb exhibit relatively uniform electron density along their long axes. Occasionally, regions of slightly increased electron opacity are seen (Figs. 1 and 2).

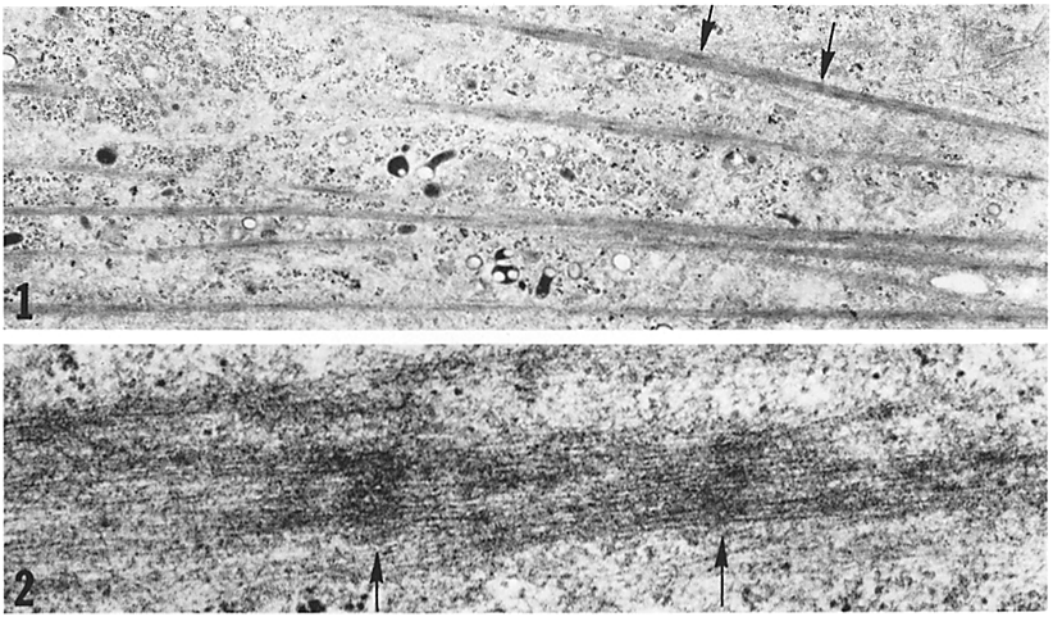
With persistent scanning of thin sections, a few areas can be found in which there are regions of greater electron density along mfb. In rare instances, alternating regions of high and low electron density can be found (Figs. 3 and 4). The lengths of the regions of higher and lower densities are not uniform, and therefore they are impossible to measure with any degree of accuracy. It should be emphasized that mfb containing areas of elevated electron density are much more difficult, and in some preparations even impossible, to locate in cells fixed without en bloc uranyl acetate staining (see Materials and Methods).

Ultrastructure of MFB After Fixation With Tannic Acid

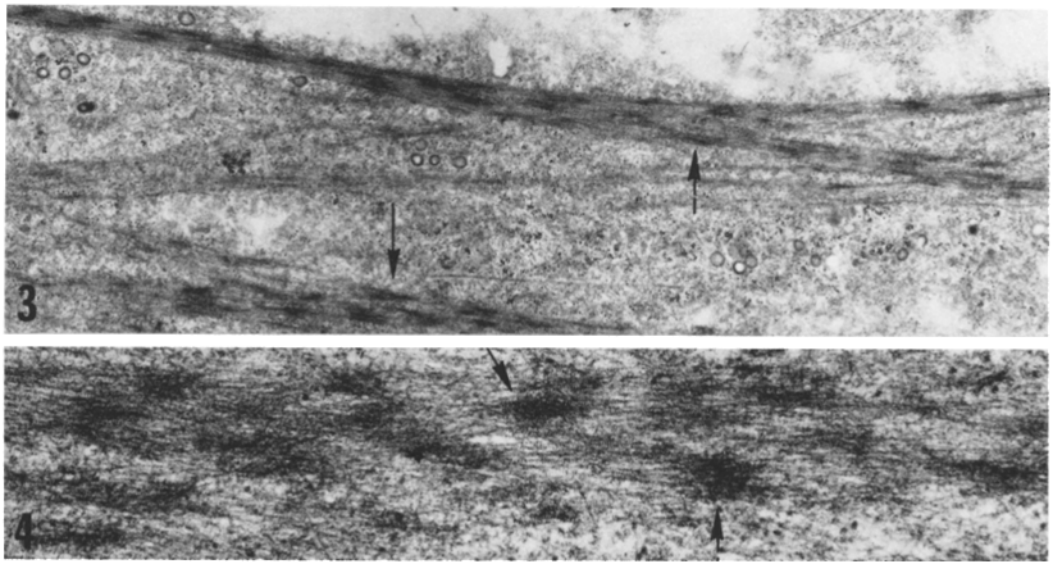
In cells fixed with the tannic acid procedure, the electron density shifts along mfb are much more obvious in all areas (Fig. 5). In many, but not all, instances, alternating electron-dense (D band) and electron-lucid (L band) areas are found (Figs. 6–8). Although a detailed ultrastructural analysis of the D bands has not been undertaken, they appear to contain some microfilaments which occasionally appear to be continuous with the microfilaments seen traversing the L bands (Figs. 7 and 8). The remainder of the D band appears to consist of relatively amorphous electron-dense material. The most apparent structures comprising the L bands are microfilaments (Figs. 7 and 8).

Comparison of D and L Bands With The Bright And Dark Zones Seen By Indirect Immunofluorescence Utilizing Myosin Antiserum

We have demonstrated alternating dark and



FIGURES 1 and 2 Low and high magnification electron micrographs showing typical BHK-21 mfb after the conventional glutaraldehyde fixation procedure and en bloc staining with uranyl acetate. Areas of increased electron density (arrows) are occasionally seen. Fig. 1, × 9,700; Fig. 2, × 48,000.



FIGURES 3 and 4 BHK-21 cells fixed with the conventional glutaraldehyde procedure. Mfb are present containing areas of greater electron density (arrows). These areas are much more difficult to locate than those seen in Figs. 1 and 2. Similar mfb are almost impossible to find when cells are fixed with glutaraldehyde and are not subjected to soaking in uranyl acetate before embedding. Fig. 3, × 11,300; Fig. 4, × 44,400.

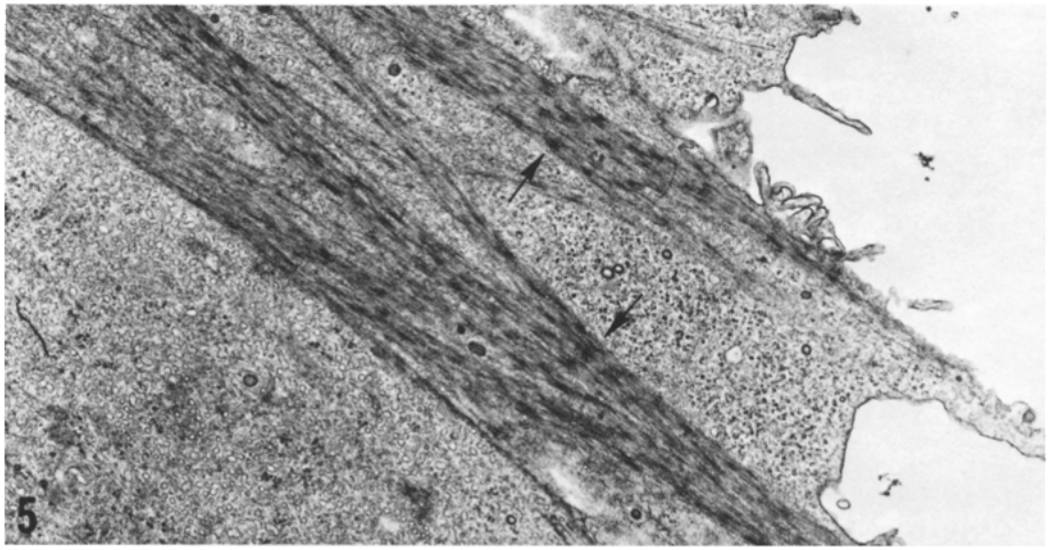


FIGURE 5 Low magnification electron micrograph of an area of a BHK-21 cell containing mfb. This cell was fixed by the glutaraldehyde-tannic acid procedure. Much more obvious alterations in electron density (arrows) are seen in mfb throughout cells prepared by this method. × 9,000.

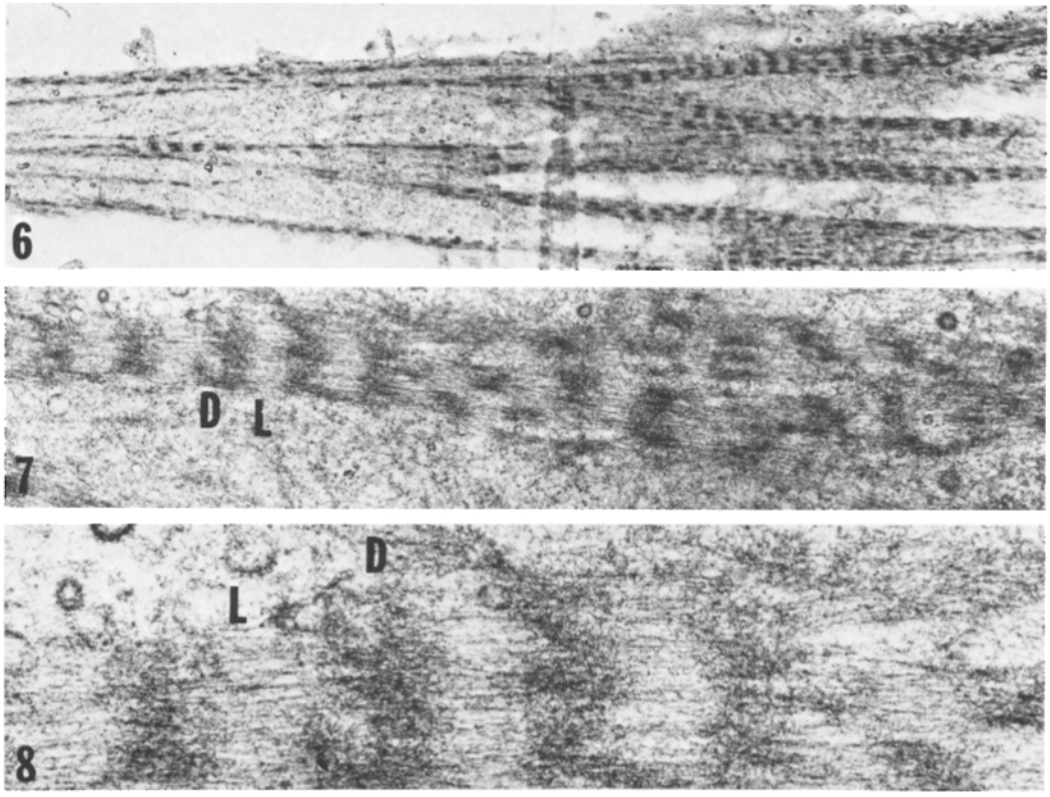
bright zones or striated patterns along many of the stress fibers in BHK-21 cells by indirect immunofluorescence with an antiserum directed against highly purified BHK-21 myosin (43). Therefore, it is of interest to determine whether or not a relationship exists between the lengths of the dark

and bright zones seen by indirect immunofluorescence and the D and L bands seen by electron microscopy after the tannic acid fixation procedure. Measurements have been obtained from fluorescence and electron micrographs selected for the most obvious and regular displays of alternating contrast. Examples of the types of microfilament bundles used for electron microscope measurements are seen in Figs. 6–8, and examples of the types of stress fibers used for light microscope measurements are seen in Fig. 9. The data for both the light and electron microscope observations are summarized in Tables I and II.

The results of a student's t test comparing the means obtained for the fluorescent (F) zones and the nonfluorescent (NF) zones (Table I) demonstrate that the mean lengths are significantly different ($p < 0.1$). Therefore, the mean length of the F zones is greater (0.59 μm) than the mean length of the NF zones (0.47 μm). When the same test is applied to the means of the lengths of D and L bands (Table II), the results indicate that the D bands (0.33 μm) are significantly longer than the L bands (0.28 μm) ($p < 0.1$).

DISCUSSION

The use of tannic acid during glutaraldehyde fixation enhances the ultrastructural detail seen within the mfb of BHK-21 cells, the most obvious features being alternating electron-dense (D) and electron-lucid (L) bands. In many instances, the D and L bands present a regular striated pattern along mfb. It should be emphasized, however, that the use of these terms does not imply a generalized periodic pattern or a uniform spacing between bands in all mfb. Indeed, there are mfb in which the D and L bands do not appear regularly spaced; nor do they traverse the entire width of mfb (see Fig. 5). The latter inconsistencies in ultrastructure may reflect the plane of the



FIGURES 6–8 Electron micrographs taken at different magnifications and BHK-21 mfb containing ordered arrays of alternating electron-dense (*D*) and electron-lucid (*L*) bands. These micrographs were obtained from cells fixed with the glutaraldehyde-tannic acid solution. Fig. 6, × 4,500; Fig. 7, × 19,000; Fig. 8, × 48,100.

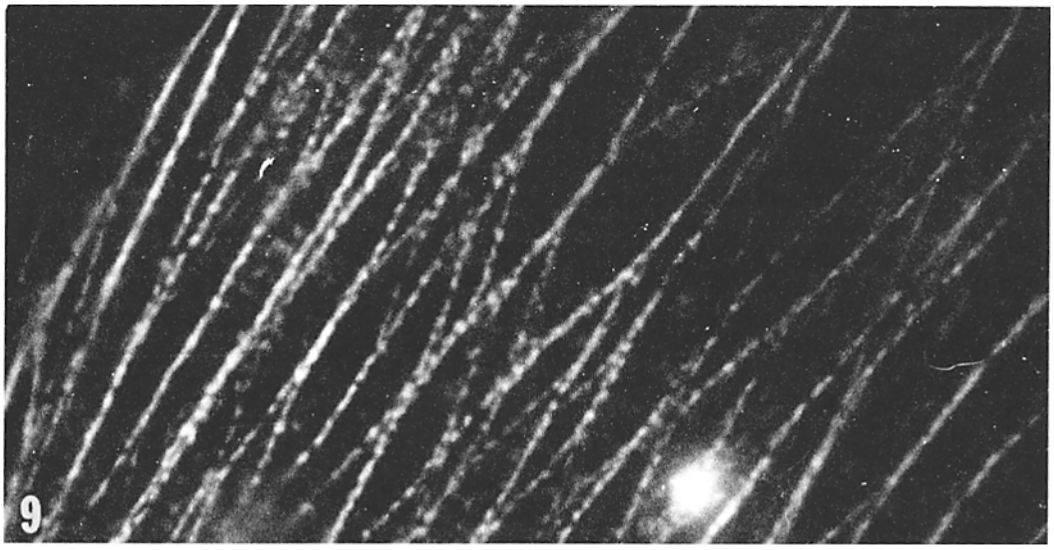


FIGURE 9 An area of a BHK-21 cell which was prepared for indirect immunofluorescence with BHK-21 myosin antiserum. 24 h before processing, cells were placed on coverslips in medium containing 0.5% calf serum, which resulted in the extreme flattening of many cells. This flattening effect provided optimal conditions for observing the stress fibers of BHK-21 cells. Note intermittent staining of stress fibers, resulting in the appearance of F and NF zones. × 2,800.

TABLE I

Lengths of Fluorescent (F) and Non-Fluorescent (NF) Zones Along BHK-21 Stress Fibers From Indirect Immunofluorescence Preparations Utilizing BHK-21 Myosin Antiserum

	n*	Mean	SD
		μm	
F zones	102	0.59	0.13
NF zones	87	0.47	0.19
Pairs (F + NF zones)	87	1.06	0.26

Lengths were determined either directly from 35-mm negatives by measurement through a dissecting microscope at ×10 or from ×3–4 enlarged positives. Measurements were equal with both techniques. A total of 19 different stress fibers from six different cells were used for the measurements.

* n = number of measurements

thin section through mfb, the variation in physiological state of individual mfb, or the merger of several bundles (12) in which the banding patterns are out of register.

The relationship between the banding patterns of BHK-21 mfb and the substructure of stress fibers as seen after myosin antiserum staining remains uncertain. The results indicate that the lengths of the fluorescent (F) and nonfluorescent (NF) zones average 0.59 μm and 0.47 μm, respectively, while the D and L bands average 0.33 μm and 0.28 μm, respectively. These data suggest that the periodicities seen by light microscopy differ from those seen by electron microscopy. It should be noted, however, that measurements at both levels of resolution are probably inaccurate. For example, it is extremely difficult to measure exactly the lengths of F and NF zones, as sharp edges are not apparent at their peripheries. In-

TABLE II

Lengths of D and L Bands as Determined From Electron Micrographs of BHK-21 Cells Fixed in Glutaraldehyde Containing Tannic Acid

	n*	Mean	SD
		μm	
D bands	61	0.33	0.07
L bands	54	0.28	0.08
Pairs (D + L bands)	54	0.62	0.12

The lengths of D and L bands were measured at final magnification of ×1,500–10,000 on either original electron microscope plates (negatives) or on enlarged prints (positives). Measurements were made by drawing a line from the mid-region of one band to the mid-region of the next band (the mid-region being approximately one half of the thickness of an mfb). A total of seven microfilament bundles in three different cells from three different preparations were measured.

* n = number of measurements

stead, there is a gradual decrease in fluorescence intensity marking the boundaries between the F and NF zones. Other errors may stem from the fact that the lengths measured on the fluorescence micrographs are approaching the theoretical limit of resolution of the light microscope.

It is equally difficult to make exact measurements on the electron micrographs because of the unevenness of the edges of D and L bands. This poses the problem of where to begin and end the measurements. Furthermore, the difficulties in making and comparing measurements from both the light and electron micrographs may also be complicated by the distinctly different modes of fixation and preparation which the cells are subjected to before actual viewing.

Let us, for a moment, consider the possibility that the overall banding patterns seen by the light and electron microscope techniques correspond to each other. Thus, for example, the longer F zones and D bands might represent the same structures. The F zones presumably contain myosin by immunofluorescence criteria, and therefore it is possible that the D bands reflect the position of myosin molecules at the ultrastructural level. The findings that the L bands are much less electron dense and appear to contain only microfilaments and that the D bands exhibit greater electron density and contain microfilaments as well as other material also hint that myosin may reside in D bands. Furthermore, the average dimensions of synthetic BHK-21 myosin thick filaments is ~0.27 μm (43). Therefore, it is certainly possible that if such thick filaments were present, they could fit within the average dimension obtained from D bands. Fujiwara and Pollard (6) have also considered that nonmuscle myosin thick filaments could fit within the fluorescent regions of human cell stress fibers which have been stained with myosin antibodies directly conjugated with fluorescein. It must be emphasized, however, that the localization of myosin at the ultrastructural level is purely speculative and based on circumstantial evidence. Myosin thick filaments could also fit within the L bands. Only an immunoelectron-microscopic approach will provide the correct answer to the specific location of myosin within mfb.

Striated mfb are not unique to BHK-21 cells. They have been reported in various cells comprising tissues which appear to be in a class which bridges the gap between muscle and nonmuscle. The most extensively studied of these cell types is the vascular endothelial cell. This cell is thought to be contractile and thereby involved in regulating the luminal space of arteries (15). It contains mfb with regularly spaced electron-dense disks ~0.1 μm wide and ~0.5–0.6 μm apart (7, 30, 44). These organelles are thought to be contractile and the disks are thought to be analogous to the Z-lines of striated muscle cells and the dense bodies of smooth muscle cells (15). However, there is no direct evidence to support either of these possibilities. Striated mfb are also seen in myoepithelial cells, and it has been suggested that they are contractile and function in glandular secretion (5). The so-called leptomeric organelles of some muscle cells also appear similar to striated mfb (18, 36), but no function has been attributed to them.

In summary, the inclusion of tannic acid appears to greatly enhance the ultrastructural detail seen in thin sections of BHK-21 mfb. The use of this procedure has not yet been thoroughly analyzed in other cell types, but preliminary observations suggest that the results will be similar. We are currently attempting to use several other antibodies which stain stress fibers periodically (e.g., α-actinin and tropomyosin) in a more in-depth approach to correlating the ultrastructural and immunofluorescence patterns. Ultimately, this should shed new light on the supramolecular organization of these proteins within mfb. This, in turn, should lead to a greater understanding of the contractile functions of these structures during various aspects of cell motility.

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