STUDIES ON THE ORGANIZATION AND LOCALIZATION OF
ACTIN AND MYOSIN IN NEURONS

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ABSTRACT

The organization of actin in mouse neuroblastoma and chicken dorsal root ganglion (DRG) nerve cells was investigated by means of a variety of electron microscope techniques. Microspikes of neuroblastoma cells contained bundles of 7- to 8-nm actin filaments which originated in the interior of the neurite. In the presence of high concentrations of Mg++ ion, filaments in these bundles became highly ordered to form paracrystals. Actin filaments, but not bundles, were observed in growth cones of DRG cells. Actin was localized in the cell body, neurites, and microspikes of both DRG and neuroblastoma nerve cells by fluorescein-labeled S1. Myosin was localized primarily in the neurites of chick DRG nerve cells with fluorescein-labeled anti-brain myosin antibody. This antibody also stained stress fibers in fibroblasts and myoblasts but did not stain muscle myofibrils.

KEY WORDS neurons microspikes microfilaments myosin localization

Neurons exhibit a variety of motile phenomena, one of the most dramatic examples being nerve outgrowth. In experiments which proved the neuron theory, Harrison (28) first described the rapid movement of nerve cells in tissue culture where growth cones of individual nerve cells advanced in an amoeboid fashion, drawing out long processes from the stationary cell body. A second neuronal process characterized by motility is axoplasmic transport. Weiss (58) originally reported that mitochondria and other organelles piled up proximal to ligated regions of axons, and that upon removal of the ligature the bulge of axoplasm moved toward the axon terminal at a rate of 1 mm/d. This result has been confirmed and, in addition, sophisticated radio-tracer experiments have identified a second transport system with rates of up to 400 mm/d (35). Energy for axoplasmic transport must be provided by each segment of the neurite, since local anoxia or treatment with metabolic poisons stops the transport and causes a piling up of material proximal to the block (44). A retrograde transport has also been described in which material moves from the axon tip toward the cell body (35). Some form of motility may also be required for transmitter release at the synapse (4).

These examples demonstrate that several nerve functions involve motility. The mechanisms responsible for these movements, however, are completely unknown. In thin sections of neurons, the most obvious structural components are the 25-nm neurotubules (microtubules) and the 10-nm neurofilaments, and it has been suggested that these components are involved in axoplasmic transport (35, 44). Microfilaments have rarely been seen in neurons in vivo, although biochemical evidence indicates that nerve cells contain substantial amounts of cytoplasmic actin (6, 22, 34, 42). Cytoplasmic myosin has also been isolated from nerve tissue and characterized, although precise location has not been determined (4, 11, 34). It is likely that these contractile proteins, perhaps in conjunction with microtu-
bules and neurofilaments, are responsible for the motile nerve functions described above.

To construct models which explain how contractile proteins interact to cause movements, it will be necessary to determine the organization and location of these proteins in the nerve cell. Although long, straight microfilaments have not been seen in central or peripheral nerve tissue prepared for electron microscopy by standard techniques, LeBeaux and Willemot (37, 38) have recently shown the presence of heavy meromyosin (HMM)-decoratable filaments in pieces of glycerinated rat caudate nucleus and substantia nigra. It is not known whether the glycerol-HMM treatment acted to induce the polymerization of actin or to protect existing filaments from disruptive actions of fixation and dehydration. Dorsal root ganglion (DRG) cells grown in tissue culture are also known to contain large amounts of actin (22), although it has been difficult to ultrastructurally identify actin filaments in these cells (13, 62). In mouse neuroblastoma cells, however, large numbers of microfilaments have been seen in cells prepared for conventional electron microscopy (48) and, in addition, these filaments have been decorated with HMM (12, 15). Myosin in nerve cells has not been localized directly, but, using cell fractionation techniques, Berl (4) has demonstrated that a substantial portion of the brain myosin is associated with the synaptic vesicle fraction.

This paper describes the arrangement of actin filaments in neuroblastoma and DRG nerve cells as determined by a variety of ultrastructural techniques, as well as by a probe specific for actin (fluorescent $S_1$). The localization of myosin in DRG cells was determined by use of fluorescently labeled antibody specific for brain myosin.

**MATERIALS AND METHODS**

**Tissue Culture**

Mouse neuroblastoma cells were grown at 37°C in a 5% CO₂ atmosphere, according to standard procedures (48). Primary cultures of chicken DRG nerve cells were prepared as described by Okun (45), and cardiac cell cultures were prepared by trypsinizing pieces of 10-d chick embryo heart for 20 min at 37°C, and using the DRG culturing solutions.

**Myofibril Preparation**

Myofibrils were prepared from adult chicken pectoral muscle by the method of Szent-Györgyi (52).

**Isolation and Fluorescent Labeling of $S_1$**

Myosin subfragment $S_1$, prepared from rabbit back muscle myosin by the method of Margossian and Lowey (39), was labeled with fluorescein by the protected method of Sanger (50) with the following modifications. Instead of 100 μg of fluorescein isothiocyanate per mg of protein, 30 μg/mg were used, and additional purification of labeled $S_1$ was achieved by ammonium sulfate fractionation (1.6–2.2 M saturated ammonium sulfate fraction). The purified fluorescent $S_1$ ($F-S_1$) was stored in 25% glycerol at -18°C. To prevent protein aggregation and to reduce nonspecific staining, bovine serum albumin (1 mg/ml) was added to the $F-S_1$ and the solution was clarified by centrifugation (35,000 g, 60 min) before cell staining.

**Isolation and Labeling of Myosin Antibodies**

Antibody was raised in rabbits as previously described (34), and immunoglobulin G (IgG) was isolated from serum by ammonium sulfate fractionation (32) and diethylaminoethyl (DEAE) chromatography (57). Purified IgG was reacted with fluorescein isothiocyanate (10 μg/mg protein) at pH 9.5 for 8 h at 4°C. After labeling, the preparation was desalted on Sephadex G-25 and then chromatographed on a DEAE cellulose column. Labeled IgG fractions obtained by step elution with increasing salt concentrations (14) were concentrated by dialysis against Aquacide II (Calbiochem, San Diego, Calif.) and stored sterilely at 4°C.

**Immunoelectrophoresis**

Immunoelectrophoresis was carried out in 1% agarose containing 20 mM sodium pyrophosphate buffer, pH 8.5 (24). A crude extract was prepared by homogenizing chick brains in an equal volume (wt/vol) of 0.6 M KCl, 20 mM sodium pyrophosphate, pH 7.0, in a glass-Teflon homogenizer. This homogenate was clarified by centrifugation (100,000 g for 60 min at 4°C), and the supernate was stored frozen in aliquots. Brain myosin was purified as previously described (34). 10 μl of the purified brain myosin (0.5 mg/ml) or crude homogenate (9.0 mg/ml) were electrophoresed in the first dimension for 1.5 h at 15 mA. The agarose gel was then removed from the apparatus, and 50 μl of antiseraum was placed in a trough adjacent to the electrophoresed protein and allowed to diffuse for 48 h at room temperature in a moist atmosphere. Finally, gels were washed overnight in 20 mM sodium pyrophosphate, washed briefly in water, and then stained for protein by the method of Fairbanks et al. (20).

**Whole-Mount Transmission Electron Microscopy (WMTEM)**

Electron microscope grids were prepared for tissue culture in the following manner: A clean glass slide was
dipped into a 0.5% solution of Formvar in ethylene dichloride and allowed to air dry. With a clean razor blade, the film surface was scored to give three 22-mm squares which were then gently floated onto a clean water surface. Several acetone-cleaned 200-mesh stainless steel grids were placed on each square, and the combination was picked up from above with a glass cover slip. A fairly heavy layer of carbon was then evaporated onto the Formvar-covered grids, and, after glow discharge treatment to render the grids hydrophilic, they were placed in a polylsine solution (1 mg/ml in H2O). After 3–4 h, the coverslip with the grids attached was washed in several changes of distilled water, transferred to a 35 x 10 mm plastic petri dish, and sterilized for 30 min under an ultraviolet light source (Sylvania germicidal no. G15T8, Sylvania Chemical Co., Millburn, N. J.), at a distance of 25 cm. Just before plating cells, the petri dishes were rinsed once with sterile culture media.

The cells, which were growing on electron microscope grids, were prepared for WMTEM as described by Buckley and Porter (10). Cells were fixed for 10 min at room temperature in 2% glutaraldehyde made up in 0.1 M KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM piperazine-N,N’-bis(2-ethane sulfonic acid) (PIPES) buffer, pH 6.9 (PMCK buffer). The cells were rinsed in buffer, post-fixed in 1% osmium tetroxide (in PMCK buffer) for 10 min, stained with uranyl acetate, dehydrated, and critical point dried exactly as described by Buckley and Porter (10). After coating with a layer of carbon, specimens were examined in a Philips 201 electron microscope operated at 80 or 100 KV.

Studies on Detergent-Treated Cells

Cells grown on stainless steel, electron microscope grids as described above were gently obtained by the method of Clarke et al. (16). The electron microscope grid was removed from the glass coverslip and held in a pair of fine forceps while a stream of PMCK buffer containing 0.01% Triton X-100 was aimed at the cells through a drawn-out Pasteur pipet. These detergent-treated cells were then either fixed and prepared for WMTEM as described above or immediately negatively stained with 1% aqueous uranyl acetate. Cells to be stained with fluorescent antibody were rinsed once with warm phosphate-buffered saline (PBS, 0.15 M NaCl, 10 mM phosphate buffer, pH 7.0) and then fixed for 10 min at room temperature in PBS containing 4% formalin and 0.15 M sucrose. The sucrose was required to prevent blebbing of the nerve cells during fixation. Fixed cells were rinsed once in PBS, placed into 100% acetone at 0°C for 10 min, and then air dried. Cells prepared in this manner could be successfully stored desiccated at -18°C for several days. Cells to be reacted with F-S1 were either treated with 0°C acetone for 1 min and air dried, or exposed to 0.01% Triton X-100 in PMCK buffer for 30–60 s and then rinsed in detergent-free buffer.

Cells prepared by one of the above-described methods were stained with the fluorescent probe for 30 min at room temperature in a humidified chamber. Unbound reagent was removed by washing in three changes of PBS over a 30-min period. The cells were rinsed briefly in distilled H2O, and then mounted in 50% glycerol containing 10 mM K+-carbonate buffer, pH 8.5, and examined with a Zeiss microscope equipped with epifluorescence or darkfield fluorescence optics. Unfixed myofibrils were stained by suspension in the fluorescent reagent and washed by low-speed centrifugations. Photomicrographs were taken on Tri-X film (Kodak) and development was carried out for 11 min in Acufine developer (Acufine, Inc., Chicago, Ill.).

To demonstrate specificity of fluorescent staining, several control experiments were performed. Staining of cells treated with F-S1 could be reversed by washing the cells with buffer containing 5 mM ATP, which served to dissociate the acto-F-S1 complex. Reversal of staining would also have been obtained, however, if the ATP solubilized the cellular actin. This was not the case since cells pretreated with 5 mM ATP retained their phase-dense stress fibers and stained normally with F-S1 after removal of ATP.

The following controls established specificity of the fluorescent antibody staining: (a) Cells treated with fluorescently labeled nonimmune serum did not stain. (b) Cells which were pre-incubated with unlabeled immune serum did not stain, while pre-incubation with unlabeled pre-immune serum had no effect on the staining. For these studies the cells were incubated in unlabeled pre-immune or immune serum (diluted 1:2 with PBS) for 30 min, washed in buffer for 5 min, and then reacted with the fluorescein-labeled immune IgG (the labeled IgG contained either immune or pre-immune serum in a ratio of one part serum to three parts labeled IgG). After a 30-min incubation, the preparation was washed and mounted as described above. (c) Antibody which had been adsorbed with antigen failed to stain the cells. Brain or breast myosin (0.2 mg) in 0.6 M KCl was added to the fluorescently labeled antiserum (0.1 mg) and then dialyzed overnight against PBS. The myosin aggregated under these conditions and could be sedimented by centrifugation, thereby removing the specific antibody. The supernate was then tested for its ability to stain cells or myofibrils.

RESULTS

Actin Localization

WMTEM Analysis of Microfilament Organization

Fibroblasts examined by the whole-mount method contained mitochondria, 25-nm microtubules and 6- to 7-nm microfilaments which paralleled the long axis of the cell, as reported by
Buckley and Porter (10). The neurites and microspikes of mouse neuroblastoma cells contained an abundance of filamentous structures. A low magnification view of a neurite with many microspikes is shown in Fig. 1a. The darkly staining central portion of the neurite contained structures which at higher magnification could be identified as microtubules on the basis of size and shape. Bundles of 6- to 7-nm microfilaments were seen to emerge from this microtubule-containing central area (arrows, Fig. 1a). At higher magnification, microfilaments in the fine neuroblastoma microspikes were wavy and appeared to form a meshwork (Fig. 1b–d). Microfilaments were also

**Figure 1** Micrographs of mouse neuroblastoma cells prepared for WMTEM. (a) Neurite containing many microspikes (s). Microfilament bundles (mfb) extend from the center of the neurite out to the tips of individual microspikes. (b) Microfilament bundles extend from microtubules (mt) at the center of the neurite. (c and d) additional examples of microspikes. Note the wavy appearance of the microfilaments. Bars, 0.5 μm. (a) × 10,600. (b) × 25,000. (c) × 90,000. (d) × 52,000.
observed in whole-mount preparations of chicken DRG nerve cells. The highly spread growth cone contained many criss-crossed microfilaments (Fig. 2), and groups of microfilaments were seen in the microspikes (insert, Fig. 2).

OBSERVATIONS OF DETERGENT-TREATED, ATTACHED CELLS

CRITICAL-POINT DRIED PREPARATIONS.
An accelerating voltage of 100 KV was insufficient to examine thicker regions of intact critical-point dried cells. To better visualize these internal structures, therefore, the upper cell membrane of attached cells was gently removed with a stream of detergent-containing buffer before fixation and critical-point drying. Fig. 3a illustrates a neuroblastoma cell treated in this manner, revealing large cables composed of microfilaments. As in intact cells, the bundles emanated from the central part of the neurite and fused to form a tight rod which filled the microspike. It was also possible to discern the individual microfilaments which joined to form the bundles (Fig. 3b).

NEGATIVELY STAINED PREPARATIONS.
To increase resolution while at the same time reducing the number of manipulations, detergent-treated cells were immediately negatively stained with uranyl acetate and examined with the electron microscope. Detergent-treated, negatively stained chicken fibroblasts contained meshworks of criss-crossing "stress fibers" and individual microfilaments (Fig. 4a). In microspike regions, microfilaments joined to form bundles, and these bundles in turn fused to form a rodlike structure in the microspike (Fig. 4b).

Fig. 5A shows part of a detergent-treated, negatively stained neuroblastoma cell. At low magnification, it was possible to see filamentous material in the interior of the neurite as well as darkly staining microspikes at the periphery. When examined at higher magnification, individual microfilaments could be recognized in these microspikes. Fig. 5B illustrates a fine microspike with its membrane partially splayed open, revealing a rod composed of tightly packed microfilaments. Figs. 5C-E depict microspikes with completely removed membranes. Bundles of straight, aligned microfilaments which sometimes appeared

**Figure 2** WMTEM preparation of DRG growth cone. Note meshwork of filaments in the growth cone which extend into the microspikes. Proximal portion of the growth cone is printed lighter to reveal intracellular organelles. (inset) High magnification of a microspike which contains microfilaments. Bars, 1.0 μm. × 4,800. (inset) × 12,500.
PARACRYSTAL FORMATION. The fact that filaments within a microspike were occasionally in register suggested the possibility of inducing paracrystals in situ. Addition of 25 mM MgCl₂ to the detergent buffer enhanced the paracrystalline order of the filament bundles (Fig. 6). The observed periodicity was ~35 nm, the same as the crossover repeat for purified actin paracrystals (34).

ACTIN LOCALIZATION WITH F-S₁
CHARACTERIZATION OF F-S₁. The fluorescein-labeled myosin subfragment S₁ (F-S₁) was shown to be a specific probe for actin by the following criteria: (a) The F-S₁ attached to purified muscle f-actin filaments to form characteristic arrowheads (Fig. 7a). Formation of these structures was prevented by washing the electron microscope grid with buffer containing 1 mM Mg-ATP. (b) Addition of F-S₁ to muscle myofibrils resulted in a distinctive staining pattern (Fig. 7b); the actin-containing "I" bands appeared as bright doublets intersected by the unstained "Z" bands, and there was no staining of the "A" bands which contained myosin. This characteristic staining pattern was prevented when the incubation was carried out in either 5 mM Mg-ATP or 5 mM Na-pyrophosphate. (c) The F-S₁ also labeled the

FIGURE 3 Electron micrographs of detergent-treated, critical point dried neuroblastoma cells. (a) Neurite with many microspikes. Microfilament bundles (mfb) fuse and insert into microspikes. (b) Higher magnification of microspikes. Note individual microfilaments (mf). Bars, 0.5 μm. (a) × 11,000. (b) × 19,000.
Electron micrographs of detergent-opened, negatively stained chicken fibroblasts. (a) Low magnification reveals large bundles of microfilaments at edge of the opened fibroblast. Note microspikes (s). Bar, 1.0 μm. (a) × 10,000. (b) Higher magnification showing individual double helical actin filaments in a microspike. Bar, 0.1 μm. (b) × 78,000.

ACTIN LOCALIZATION IN NERVE CELLS.

Mouse neuroblastoma cells reacted with F-S1 exhibited a diffuse staining throughout the perinuclear region and neurites. In addition, actin-containing microspikes stained intensely (Fig. 8A and B). If the reaction was carried out with ATP, the microspikes no longer stained, and fluorescence in the cell body was greatly reduced.

The distribution of actin was also investigated in chicken DRG nerve cultures. In addition to nerve cells, these cultures usually contained a small number of fibroblast-like satellite cells. As expected, the actin-containing stress fibers in these satellite cells stained specifically with the F-S1 (see Fig. 7c). DRG nerve cells, on the other hand, revealed no discrete fluorescent localization. Instead, the neurites and growth cone regions were stained uniformly (Fig. 8C and D). Localization in these structures was specific, however, because this staining pattern was not seen in the control which was washed with ATP-containing buffer.

Myosin Localization

CHARACTERIZATION OF MYOSIN ANTIBODIES

Antibodies to brain or breast myosin were tested against antigen by the Ouchterlony double-diffusion technique. The antibody to chicken brain myosin reacted strongly with purified chicken
brain myosin but did not react with myosin from chicken gizzard or breast, or rabbit back muscle. Antibody to chicken breast muscle myosin gave a reaction with chicken striated muscle myosin only. No reaction of chicken breast antimyosin was seen with chicken brain or gizzard myosin, or with rabbit striated muscle myosin (see Fig. 13 of reference 34). The antibody to brain myosin was further characterized by immunoelectrophoresis. A single precipitin arc was obtained for the pure

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\caption{Electron micrographs of detergent-opened, negatively stained neuroblastoma cell. (A) Large neurite with several microspikes (s). Bar, 1.0 \( \mu \text{m}. \) (B-E) Higher magnification of A, showing detail of microspikes. (B) microspike with membrane partially removed, exposing a microfilament bundle. (C, D, and E) microspikes with membranes completely removed to reveal bundles of actin filaments. Bars, 0.1 \( \mu \text{m}. \) (A) \( \times 12,000. \) (B) \( \times 51,000. \) (C) \( \times 40,000. \) (D) \( \times 110,000. \) (E) \( \times 95,000. \) }
\end{figure}
myosin (Fig. 9a). In the reaction with the crude homogenate, a single precipitin line was also obtained (Fig. 9b), indicating that the antibody was monospecific.

Fluorescently labeled antibodies were also tested for specificity by staining isolated breast muscle myofibrils. Fluorescently labeled antibody to brain myosin did not stain myofibrils (Fig. 10a and b). The antibody to breast muscle myosin, however, did stain the myosin-containing "A" bands of the myofibrils (Fig. 10c and d). This staining pattern was abolished when the immune serum was pre-adsorbed with chicken striated muscle myosin. Staining was not affected, however, by pre-adsorbing the serum with chicken gizzard or brain myosin (data not shown).

**MYOSIN LOCALIZATION WITH FLUORESCENTLY LABELED MYOSIN ANTIBODY**

Fluorescently labeled antibody to nerve myosin (obtained from the chicken brain) was used to stain cultured nerve cells of the chicken dorsal root ganglion. Staining was diffuse and not localized to any particular region of the neuron. Fluorescent staining occurred in the growth cone, neurite, and cell body but not in the nucleus (Fig. 11 C–F). This diffuse staining of the nerve cells could be prevented by blocking the reaction with unlabeled immune serum. Satellite cells which were present in the nerve cultures also stained with the antibody to brain myosin. Fluorescent staining was localized in the stress fibers of these cells (Fig. 11 A and B). Often, the staining occurred in discrete patches along the length of a stress fiber, giving rise to regular periodicities. The antibody to chick brain myosin did not stain stress fibers of mouse neuroblastoma, A-9, or primary fibroblast cells. Likewise, human macrophages, HeLa, or MRC-5 cells did not stain. Stress fibers in baby hamster kidney (BHK) cells did, however, exhibit distinct staining with the chicken antibody. The significance of these interspecific differences is not obvious.

To further demonstrate specificity of the brain myosin antibody, various control experiments were carried out, with the characteristic staining of fibroblast stress fibers as a test system. Large numbers of fibroblasts, as well as some myoblasts, were obtained from 10-d-old chick embryo hearts. In one study, cardiac cell cultures were pre-incubated with either pre-immune or immune serum before staining with fluorescently labeled anti-brain myosin antibody. The unlabeled immune serum prevented staining of fibroblast stress fibers (Fig. 12a and b), while the pre-immune serum did not affect the staining (Fig. 12c and d). It was also possible to prevent staining of fibroblast stress fibers by adsorbing the antibody with antigen. Thus, the anti-brain myosin anti-body which had been adsorbed with the brain myosin antigen failed to stain the stress fibers in the fibroblasts (Fig. 12g and h). However, the brain myosin antibody which had been adsorbed with breast myosin did stain the stress fibers (Fig. 12e and f), indicating that the antibody was specific for brain
by the fact that myofibrils within the cardiac cells never exhibited staining of the myosin-containing "A" bands. This characteristic type of staining could, however, be obtained by reacting the cardiac cells with fluorescently labeled antibody to striated muscle myosin (from chicken breast). This antibody stained the cardiac muscle cells brightly, and the characteristic banding pattern resulting from staining of myofibril "A" bands could be seen (Fig. 13 C and D). In contrast, the cardiac fibroblasts present in this preparation were not stained by the antibody to striated muscle myosin.

myosin and, further, that removal of antibody by brain myosin was not caused by nonspecific binding to myosin protein.

Not only fibroblasts, but also myoblasts of the cardiac cell cultures stained with anti-brain myosin antibody. It was not always possible to morphologically identify myoblasts, but occasionally myofibrils within these cells could be recognized with phase contrast optics. Staining of such cells with fluorescent anti-brain myosin antibody was often diffuse, although stress fibers similar to those seen in fibroblasts were sometimes seen (Fig. 13 A and B). The fact that the cardiac muscle cells in culture stained with anti-brain myosin suggested that either the antibody cross-reacted with cardiac muscle myosin or, more likely, that the cardiac muscle cells contained both cytoplasmic and muscle myosins. This latter possibility was supported by the fact that myofibrils within the cardiac cells never exhibited staining of the myosin-containing "A" bands.
DISCUSSION

**Actin Localization**

**LOCALIZATION WITH WMTEM.** Thin sectioning of individual nerve cells is a tedious process and provides only limited information on filaments which course in and out of the plane of section. To study the localization and organization of actin in cultured nerve cells, therefore, new approaches which avoided some of these problems were used. One such method was that developed by Buckley and Porter in which whole cells were studied in the transmission electron microscope after positive staining and critical-point drying (10). Because the cells retained their three-dimensional structure, it was possible to follow individual filaments over long distances, and to trace their course by use of stereo pairs of electron micrographs. With this technique, the filament organization in thinner regions of cultured cells was investigated with a standard transmission electron microscope operated at 100 KV.

Thin-sectioned fibroblasts contain arrays of linearly arranged microfilaments (known as stress fibers [9], sheath [59] or microfilament bundles [25]) which are in close association with the basal membrane, and serial sectioning reveals that similar structures can sometimes be found near the upper membrane (26, 59). Microfilaments in these bundles bind HMM (26), indicating that they are composed of actin. In ruffling membrane regions of fibroblasts, microfilaments do not form stress fibers but rather are seen as a loose meshwork or lattice (59). Both stress fibers and three-dimensional lattice formations were maintained in chicken fibroblasts observed by the whole-mount method described above.

The highly organized microfilament bundles (stress fibers) characteristic of fibroblasts have not been seen in thin sections of cultured DRG cells (references 13, 62, and our unpublished observations). Instead, microfilaments are organized in a meshwork or lattice arrangement similar to that seen in the ruffling membrane region of fibroblasts (62). Likewise, stress fibers were not seen in DRG cells examined by WMTEM, although individual microfilaments were observed in the thin, well-spread growth cones (Fig. 2). Microfilaments which entered microspikes present at the periphery of the growth cone did, however, associate into loose bundles (Fig. 2, inset). In addition, microfilaments which were randomly distributed throughout the growth cones converged at, and appeared to continue into, the large axon which connected the growth cones to the cell body. However, the organization of filaments in the compact neurite could not be determined by this technique because of the thickness of the structure.

Burton and Kirkland (12) first showed that mouse neuroblastoma cells contained microfilaments which could be decorated by HMM and, with a modified glycerination procedure, Chang and Goldman (15) subsequently demonstrated that HMM-decorated filaments were present in all regions of the neuroblastoma cell, including the cell body, neurites, and microspikes. In a detailed ultrastructural study Ross et al. (48) described the organization of these microfilaments in neuroblastoma cells. Particularly interesting was the report that microspikes contained large numbers of mi-
FIGURE 11 Cells cultured from chick dorsal root ganglia stained with fluorescently labeled anti-brain myosin antibody. (A and B) Stress fibers in fibroblasts stained specifically with the antibody. Periodic staining can occasionally be seen along the length of a fiber (arrow). \( \times 3,100.\) (C-F) Neurites and growth cones of DRG nerve cells stained uniformly with the anti-brain myosin antibody. \( \times 1,440.\)

Microfilaments which emanated from the main neurite and sometimes appeared to be spatially associated with the neurite microtubules (see Fig. 20 of reference 48). Similar results were obtained in the present study with the whole-mount procedure (Fig. 1). Because of the depth of field available with this method, it was possible to discern the overall organization of microfilaments in a manner not possible with thin-sectioning techniques. In the whole-mount preparation, microfilaments which extended from the microtubule region were organized into bundles which joined with similar bundles present at the cell margin, forming a single structure which inserted into the microspike (Fig. 1). It was possible to obtain a clearer view of this microfilament organization by gently removing the plasma membrane before processing for WMTEM (Fig. 3). In contrast to preparations of intact neuroblastoma cells, microfilament bundles in the detergent-treated cells seemed to be more intact and to stain more darkly. While this appearance may have resulted from partial collapse of the bundles as a result of detergent treatment, it is likely that the absence of a membrane permeability barrier allowed better fixation as well as a less turbulent CO\(_2\) substitution during the critical-point drying process, resulting in the observed morphology. The fact that the microfilament bundles were not removed by the detergent treatment suggested a firm attachment to the lower cell membrane.

**Actin in Microspikes.** At the tip of a growing nerve axon is a flattened region known as the "growth cone" from which extend long, rigid microspikes (28) having a constant diameter of between 0.1 and 0.2 \( \mu \text{m} \) (8). These dynamic structures, which can grow outwards as far as 25 \( \mu \text{m} \) in 1 min, have been seen to wave about in the medium or along the solid substratum (59). Microspikes are also found in fibroblasts and other cell types (2), and are believed to be involved in cell-cell interactions (23), membrane spreading (61), exploration of the cellular environment, and cell adhesion (1). Ultrastructural studies have shown the microspike to be composed of an elongated network of microfilaments (2, 48, 59). Microtubules and 10-nm filaments are not present in the microspikes (2, 48, 59), and recent immunofluorescent localization studies indicate that myosin is also absent from the microspikes (24).

Microspikes of detergent-opened, negatively-stained neuroblastoma cells contained straight microfilaments aligned in parallel arrays (Fig. 5), and a similar organization of microfilaments was seen in fibroblast microspikes (Fig. 4). Similar results have been reported for *Dictyostelium* and sea urchin cells (16, 19). The microfilament bundles did not just fill the microspike, but extended...
FIGURE 12 Demonstration of specificity of anti-brain myosin antibody using chicken heart fibroblasts. Phase micrograph on left, corresponding fluorescent micrograph on right. (a, b) antibody staining was blocked by pre-incubating with immune serum. (c, d) staining was not affected by pre-incubating with pre-immune serum. (e, f) cells were stained by antiserum which had been adsorbed with breast myosin, but in g and h it can be seen that cells did not stain when the antiserum was adsorbed with brain myosin. × 1,250.

back into the cell for a considerable distance, and branched to form smaller bundles (Figs. 1 and 3). These bundles appeared to be firmly attached to the lower cell membrane. This type of bundle arrangement is likely to be responsible for both the rigidity and motility of the micropike. Indeed, a model has been proposed in which movement results from the interaction of myosin with actin filaments in the microvilli of intestinal brush-border cells (41).

The straight, intact microfilaments revealed in negatively stained neuroblastoma cells differed from the wavy, network image obtained by either thin section (15, 48) or WMTM (Fig. 1). This suggests that processes involved in preparing the cells for electron microscopy by the latter procedures may have disrupted filament organization. Indeed, Maupin-Szamier and Pollard (40) have shown that purified, in vitro assembled actin, when fixed in osmium tetroxide, exhibits reduced viscosity and appears broken and wavy when examined in the electron microscope. Furthermore, these authors have also shown that low concentrations of osmium tetroxide rapidly cleave the actin molecule to produce lower molecular weight species.

LOCALIZATION OF ACTIN USING F-S1. To rapidly localize actin and study its distribution within an entire cell, the fluorescent S1 probe for actin was used with both neuroblastoma and DRG nerve cells. As already noted, microfilaments were present in the neuroblastoma cell body, neurites, and micropikes (references 15, 48 and Figs. 1, 3, and 5). These same regions reacted specifically with F-S1, and the most intense stain was seen in the micropikes which contained high concentrations of actin (Fig. 8). Sanger (51) has also localized actin in neuroblastoma cells using fluorescent HMM. In DRG nerve cells, the neurites and growth cones were labeled with F-S1 (Fig. 8), confirming the location of actin as determined by ultrastructural methods (reference 62 and Fig. 2).

Although neuroblastoma cells, DRG cells, and brain tissue are known to contain significant amounts of actin, it is only in neuroblastoma cells that typical 6-nm actin filaments can readily be seen in thin section. Ultrastructural studies of DRG nerve cells revealed short 6-nm filaments
which formed a meshwork in the growth cone, and only nondescript filamentous material adjacent to the membrane of the neurite (62). In numerous electron microscope investigations of nervous tissue, few, if any, 6-nm filaments have been visualized (5, 47), although LeBeaux (36) claims to have seen a few short microfilaments in specially fixed nerve tissue, and an interesting array of filamentous meshworks is present in close association with the membrane of the Purkinje cell axon hillock (47).

Recently, LeBeaux and Willemot (37, 38) have reported the presence of large numbers of actin filaments in neurons of the rat substantia nigra and caudate nucleus. For these studies, small pieces of brain were incubated in a glycerol solution to render the cells permeable to the HMM probe used to identify actin filaments. Decorated filaments were found in both axons and dendrites, as well as in association with pre- and postsynaptic membranes. In control preparations which were either incubated in glycerol-containing buffer or incubated with HMM in the presence of ATP, undecorated actin filaments were visible. It is interesting to note that cells which had been exposed to HMM contained many more filaments than cells which were incubated in buffer plus glycerol only (37). It is known that HMM stimulates the polymerization of actin (17, 33, 63), and it is possible that glycerol, like sucrose (46), also influences the state of actin polymerization. If, indeed, neuronal actin is normally in a nonfilamentous form, this would explain why actin filaments are so rarely seen in this tissue. Evidence for membrane-associated nonfilamentous actin has been obtained in other systems (27, 54, 55).

**Myosin Localization**

Myosin cannot readily be visualized by ultrastructural means, although filaments thought to be composed of myosin have been seen in some cells (3, 43, 53). In the present study, therefore, antibody specific for myosin from chick brain was used to localize myosin in chicken DRG nerve cells with fluorescence techniques. The antibody was shown to be specific for brain myosin by Ouchterlony double-diffusion and immunoelectrophoresis. The neurites and growth cones of
DRG nerve cells stained specifically with the fluorescent antibrain myosin antibody (Fig. 11 E and F). This staining pattern was not seen when the cells were reacted with fluorescently-labeled nonimmune serum, or when the reaction was blocked by unlabeled immune serum. These results indicated that myosin was present in the nerve cell but that no discrete localization within any particular region or structure of the cell could be determined with the resolution provided by light microscopy. Because actin, like myosin, was found throughout the neuron (Fig. 8), it is possible that they may interact to produce force.

Possible functions for the myosin include movement of the growth cone and axoplasmic transport. During nerve growth, the cell body and neurite remain stationary while the growth cone "crawls" forward over the substratum, often at rates of up to 50 μm/h (28). The neurite does not grow from the base, but rather new material appears to be added at the growing tip (7). Membrane, proteins, and other materials synthesized in the cell body are transported along the neurite to the growth cone via axoplasmic transport, an active process which also may require myosin (4, 35, 44). In radiotracer experiments, Lasek and Hoffman (35) have demonstrated that five proteins make up the majority of the slow component. Two of these polypeptides appear to be tubulin, while the remaining three (68,000, 145,000, and 200,000 daltons) are thought to be associated with neurofilaments. These researchers propose a model in which the 200,000-dalton component (possibly myosin) interacts with actin attached to the axonal membrane to move the neurofilament-microtubule assembly down the axon at 1 mm/d. Ochs (44) has invoked a similar actomyosin mechanism to explain the fast transport (400–500 mm/d) of proteins and particulate matter.

The present study demonstrated that non-neuronal cells were stained by the antibody to brain myosin; large stress fibers in the satellite cells and fibroblasts present in DRG and cardiac cultures were stained by the fluorescent brain myosin antibody (Figs. 11 A and B, and 12). Myosin staining along the length of a stress fiber was often periodic, giving rise to a striated appearance. Similar patterns have been reported for other nonmuscle cells stained with anti-myosin antibody (24, 56).

The anti-brain myosin antibody, in addition to staining stress fibers in fibroblastic cells of dorsal root and cardiac tissue cultures, also stained cardiac myoblasts. Myoblasts exhibited both diffuse staining as well as staining of stress fibers. Myofibrils present in the cardiac myoblasts did not react with the antibody to brain myosin, although they could be stained by fluorescently labeled antibody to chicken skeletal muscle myosin. Holtzer et al. (29) were also able to stain chick cardiac myoblasts with antibody to chicken skeletal muscle myosin, and others have demonstrated immunological cross-reactivity between chicken skeletal and cardiac myosins (18, 21, 49). The fact that cardiac myoblasts were stained with the antibody to brain myosin but that myosin in the myofibrils of these same cells did not stain suggests that the myoblasts contain both cytoplasmic and muscle myosins. Studies on prefusion skeletal muscle myoblasts also suggest the presence of two types of myosin in the same cell (30, 31). Actin provides another example of both cytoplasmic and skeletal forms of a contractile protein being present in a cell at the same time (60).

The fact that both nerve and satellite cells stained with the antibody to brain myosin suggested either that (a) a large amount of the myosin isolated from the brain came from Schwann cells, glial cells, astrocytes, and other non-neuronal cells or that (b) the cytoplasmic myosins present in nerve and fibroblast cells are immunologically similar. It should be possible to distinguish between these two choices by using existing techniques.

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