Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4364–4368, September 1978
Cell Biology

Differences among 100-Å filament subunits from different cell types

(intermediate filaments/neurofilaments/immunofluorescence/Colcemid/cytochalasin B)

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Communicated by Britton Chance, June 12, 1978

ABSTRACT **The protein subunit of 100-Å filaments constitutes approximately 50% of the cytoskeleton protein of chick fibroblasts. In addition to the 43,000-dalton protein (constitutive actin) common to all cell types, fibroblast cytoskeletons contain a 58,000-dalton protein likely to be the 100-Å filament subunit, whereas smooth muscle contains, instead, a 55,000-dalton protein. Additional differences among 100-Å filaments are shown by immunofluorescence using antibodies against chick fibroblast 58,000-dalton component (anti-F58K) and against chick brain 100-Å filament subunits (anti-BF). Anti-F58K binds to 100-Å filaments in chick fibroblasts, presumptive myoblasts, chondroblasts, pigment cells, and neurons, but not to 100-Å filaments in mouse or human fibroblasts. This antibody stains cables of 100-Å filaments induced by sequentially treating cells with cytochalasin B and Colcemid. Anti-BF binds only to neurofilaments and not to 100-Å filaments of other cell types studied. Absorption of antibodies with purified subunits from gizzard 100-Å filaments eliminates binding of anti-F58K to the filaments of all cell types but does not diminish binding of anti-BF to neurofilaments. Various IgGs also bind nonspecifically to induced cables of 100-Å filaments. The problem of nonspecific binding of labeled antibodies, as well as the problem of cell and species specificity of the 100-Å filaments, is discussed.**

Ishikawa *et al.* (1, 2) reported that many cells display two types of metastable filaments. One set, approximately 60 Å in diameter, can be decorated with heavy meromyosin, is clustered into "stress fibers," and is associated with irregular "dense bodies" (3). Cytochalasin B (CB) partially disrupts these bundles of microfilaments. This observation led to the suggestion that microfilaments mediate contractile activities (4). Others, however, have emphasized that CB has no effect on the synthesis, assembly, or function of actin filaments in embryonic myoblasts (5, 6), whereas it induces contraction, blebbing, and arborization in many nonmyogenic cells (6–8). Therefore it is equally likely that the microfilaments subserve a skeletal function (5, 8, 9).

The filaments of the second set were termed "intermediate-sized filaments" (1). These 100-Å filaments cannot be decorated with heavy meromyosin, and course throughout the cytoplasm as randomly oriented isolated filaments, or in small aggregates of parallel filaments. Often the 100-Å filaments insert into the cell membrane, particularly in regions lacking stress fibers (10). They are present in embryonic myogenic, fibrogenic, chondrogenic, and melanogenic cells, as well as in mature nerve, smooth muscle, glia, bone, endothelial cells, and macrophages (11–19). Their intracellular aggregation is stimulated by Colcemid (1, 5, 16–18) and is further enhanced when the cells are sequentially exposed to CB and then to Colcemid (5, 8). These immense aggregates of 100-Å filaments are visible in living fibroblasts as tortuous, phase-lucent "cables" (5), which may exceed 5 μm in diameter and occupy over 20% of the cell volume.

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The induced cables of 100-Å filaments could be partially isolated in a discontinuous sucrose gradient, and such preparations consisted largely of a 58,000- and a 43,000-dalton protein (7). In this report we extend our analysis to a comparison of the 100-Å filaments in different cell types, and demonstrate, contrary to recent immunofluorescence results of others (16, 20, 21), considerable polymorphism among 100-Å filaments from different cell types and from different species.

MATERIALS AND METHODS

Cell Cultures. Cables of 100-Å filaments were induced in primary cultures of chick presumptive myoblasts and fibroblasts. The cells were treated sequentially with CB at 5 μg/ml for 48 hr, then with 1 μM Colcemid for 24–48 hr (5). Other chick embryonic cells were cultured by using similar procedures. Dorsal root ganglion cells and spinal cord cells were treated with 5-fluoro-2′-deoxyuridine to reduce nonneural cells.

Preparation and Analysis of Cytoskeletons. Two procedures were used. (*i*) High-salt insoluble residue (11, 12): Tissues or cultured cells were washed, homogenized, and extracted successively in 0.6 M KCl and 0.6 M KI in 10 mM sodium phosphate (pH 6.8) at 4° for 48–72 hr. The insoluble residue was separated by centrifugation at 16,000 × *g* for 10 min. (*ii*) Triton X-100-insoluble residue (19): Cultured cells were homogenized in 1% (vol/vol) Triton X-100 in 75 mM NaCl, 5 mM sodium phosphate (pH 6.8) for 5 min at 21°, and the insoluble material was collected by centrifugation at 2000 × *g* for 7 min. The insoluble material from both procedures will be referred to as "crude cytoskeletal preparation." The protein composition was analyzed by electrophoresis on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels (23).

Preparation of 100-Å Filament Subunits and Production of Antisera. Fibroblast cytoskeletons were obtained with Triton X-100 and applied to preparative $NaDodSO_4$/polyacrylamide gels. The band at 58,000 daltons was cut out and used to immunize rabbits. The resulting antiserum, hereafter designated "anti-F58K," to be characterized in detail elsewhere, reacted positively in Ouchterlony tests against fibroblast cytoskeletons and whole fibroblast homogenate (J. Otto, G. Bloom, and J. Bryan).

Chicken gizzard intermediate filaments were prepared according to the procedure of Small and Sobieszek (12).

A preparation of brain filament subunits was obtained from whole chicken brain by hydroxyapetite adsorption (24) and consisted of a major component of 55,000 daltons and a minor component of 51,000 daltons. Because these proteins, eluted from a preparative $NaDodSO_4$/polyacrylamide gel and injected into rabbits, proved to be poor antigens, the whole preparation was cleaved with CNBr to enhance antigenicity (24). The resulting peptide mixture (ranging between 2000 and

Abbreviations: CB, cytochalasin B; $NaDodSO_4$, sodium dodecyl sulfate.

13,000 daltons) elicited an antiserum in rabbits, hereafter designated "anti-BF," that yielded a precipitin band in immunodiffusion against chick brain homogenate, but not against chick fibroblast homogenate or purified chick brain tubulin.

Immunofluorescence Histochemistry. Cells on glass coverslips were washed three times with balanced salt solution, fixed with 2% formaldehyde in pH 7.3 phosphate-buffered saline for 7 min at 22°, followed by three rinses in phosphate-buffered saline, then placed in acetone at −20° for 10 min and air dried. Occasionally cells were fixed in methanol for 5 min at −20°, followed by a brief rinse in acetone at −20°, and air dried.

The indirect method of immunofluorescent staining was used. Immune or nonspecific rabbit serum or IgG fractions in phosphate-buffered saline were applied to cells at concentrations of 0.5–1.0 mg/ml and incubated for 60 min at 22° in humid chambers. The cells were rinsed in several changes of phosphate-buffered saline for 60 min, incubated with fluorescein-labeled goat anti-rabbit IgG (Antibodies, Inc., Davis, CA) at 0.4–1.0 mg/ml in phosphate-buffered saline for 60 min and again rinsed. After a rinse in distilled H_2O, coverslips were mounted in 50% (vol/vol) glycerol/50% 0.1 M K_2HPO_4 at pH 8.7 and examined under epi-illumination in a Zeiss fluorescence microscope.

RESULTS

Differences in the Cytoskeletons of Different Cell Types. High-salt residues of fibroblasts, smooth muscle, and brain yielded similar polypeptide patterns. Such cytoskeletons consist largely of two proteins: a 43,000-dalton protein and a protein in the 54,000- to 58,000-dalton range (Fig. 1). The 43,000-dalton component comigrates with actin. The larger component is very likely the 100-Å filament subunit (7, 11, 12, 19, 25). The 43,000- and 54,000- to 58,000-dalton polypeptides are also the major components in fibroblast, smooth muscle, and brain cytoskeletons prepared by Triton X-100. The patterns of the latter two cell types, following Triton X-100, are more complex than patterns with KI extraction. Greater than 85% of the 58,000-dalton protein is retained as a cytoskeleton component after extraction of fibroblasts with Triton X-100.

The 54,000–58,000/actin ratio varies from roughly 0.7 to 1.5 among the different cell types (Fig. 1), and there is also variation in this ratio in cultures of the same cell type, depending upon cell density. Between 70 and 90% of the cytoskeleton of both fibroblasts and smooth muscle cells consists of a 43,000-dalton protein plus one approximating 58,000 daltons. Fig. 1 also demonstrates that the protein from fibroblasts has an apparent molecular weight of 58,000, whereas that from smooth muscle is approximately 55,000. Given the extent of these cables, it is inappropriate to refer to them as "nuclear caps" (25).

Immunofluorescent Localization of Anti-F58K in Fibroblasts. In all normal chick fibroblasts, the antibody localizes to elongated filaments of various diameters (Fig. 2*A*). Fig. 2 *B* and *C* illustrate the binding of anti-F58K to cables formed in fibroblasts exposed to CB and then to Colcemid. Fibroblasts placed directly into Colcemid assemble cables exceeding 1.5 μm in diameter within 2 hr. Cells that have been pretreated with CB exhibit cables within 1 hr.

Immunological Specificity of the Anti-F58K Staining. To determine that the localization of the anti-F58K represents a specific antigen–antibody reaction rather than nonspecific IgG interactions with intracellular macromolecules (26–28), several controls were performed. Whole serum and IgG fractions at 0.1–3 mg/ml from different rabbits were used. Sera were from nonimmunized rabbits and from rabbits immunized against chick smooth muscle tropomyosin, chick skeletal myosin heavy chains, and bovine serum albumin. These sera all will be termed "nonspecific" to distinguish them as a group from anti-F58K and anti-BF. With untreated chick fibroblasts, none of these sera yielded a pattern similar to that in Fig. 2*A*.

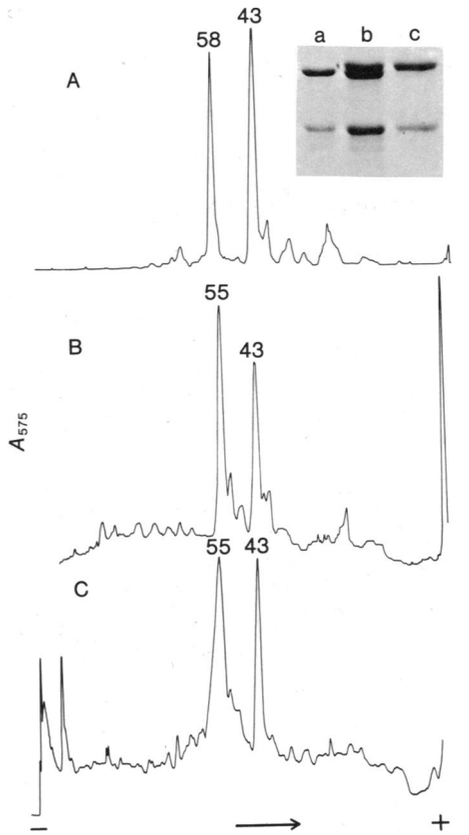

FIG. 1. Scans of partially purified cytoskeletons from (*A*) cultured fibroblasts, (*B*) gizzard smooth muscle, and (*C*) whole brain. High-salt-insoluble residues were solubilized in a $NaDodSO_4$ buffer and electrophoresed in 10% polyacrylamide cylinder gels. The major components of each preparation are polypeptides of approximately 55,000–58,000 and 43,000 daltons. (*Inset*) Slab gel electrophoresis of cytoskeletal proteins of (a) gizzard, (b) fibroblasts and gizzard combined, and (c) fibroblasts. Note that the larger components of the two cell types do not comigrate. Their molecular weights approximate 58,000 in the fibroblast preparation and 55,000 in the gizzard preparation. The major brain cytoskeletal proteins extend from approximately 51,000 to 55,000.

In striking contrast, virtually all nonspecific sera and IgG fractions, when used at high concentrations, stained the cables in fibroblasts treated with CB and Colcemid (Fig. 2*D*). For the following reasons we do not believe that the staining of the cables with nonspecific IgG represents an antigen–antibody reaction: (*i*) Staining of the induced cables by the nonspecific IgG was obtained only with high concentrations. Moderate staining was observed at 3 mg/ml, and virtually no staining at 0.5 mg/ml. (*ii*) Nonspecific IgG did not stain, even at the highest concentrations (3 mg/ml), the fine filaments that are shown in Fig. 2*A*. The anti-F58K IgG stains the fine filaments in untreated fibroblasts, as well as the cables, intensely, at concentrations as low as 0.1 mg/ml. (*iii*) Antigen–antibody complexes, but not nonspecific antibody binding, resist dissociation in isotonic salt solutions at high pH. There was no diminution in staining intensity of untreated or CB/Colcemid-treated fibroblasts if, after incubation with anti-F58K, they were washed for 30 min in saline buffered to pH 9.5 before incubation with labeled goat anti-rabbit IgG. In contrast, the binding of nonspecific IgG to the induced cables is virtually abolished after this treatment.

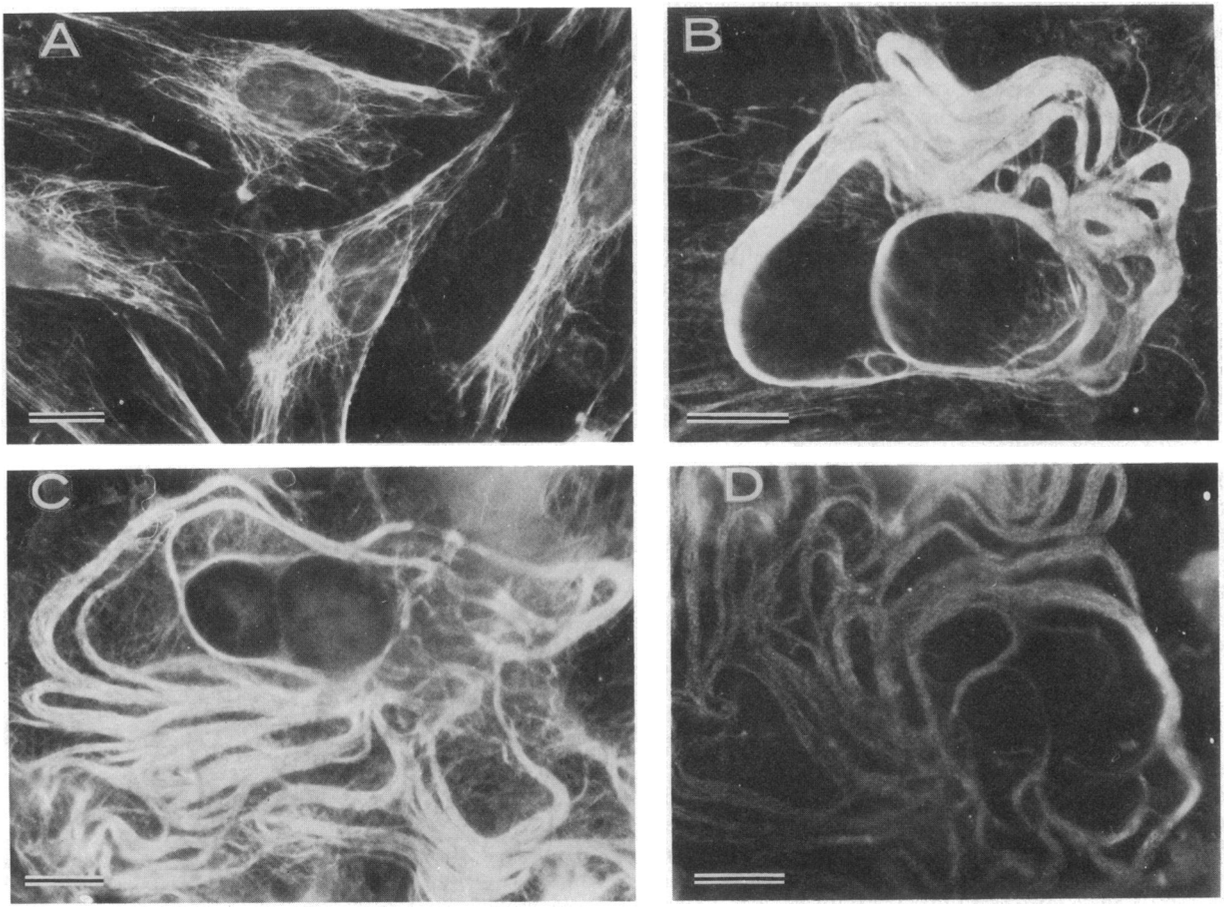

FIG. 2. Fluorescence micrographs of chick fibroblasts incubated with anti-F58K at 0.5 mg/ml (*A–C*) or with nonspecific IgG at 1 mg/ml (*D*). (*A*) Untreated cells; (*B–D*) CB/Colcemid-treated cells. In untreated fibroblasts, anti-F58K is bound to filaments of various thickness and distribution, which extend into the finest processes. It is to be stressed that nonspecific IgG did not bind to the fine filaments of untreated fibroblasts under any conditions. In CB/Colcemid-treated cells, anti-F58K is bound primarily to the massive cables whose conformation and compactness varies greatly from cell to cell. Extraction of untreated or CB/Colcemid-treated cells with Triton X-100 prior to fixation (i.e., cytoskeletons) and incubation with anti-F58K resulted in similar immunofluorescence patterns. The cell in *C* was washed at pH 9.5 after incubation with anti-F58K. (*D*) A CB/Colcemid-treated fibroblast stained with a nonspecific IgG. The exposure time for this micrograph was twice that for *B* and *C*, and the cell was selected for its atypical conspicuous fluorescence. When cells incubated with nonspecific IgG are washed at pH 9.5, fluorescent cables are not sufficiently visible for photography. (Bar = 15 μm.)

Anti-F58K Staining of the 100-Å Filaments in Different Chick Cell Types. Various chick cells were cultured and processed with the anti-F58K. The anti-F58K stained a filament network in cultured myogenic, retinal pigment, gizzard, and chondrogenic cells. CB/Colcemid cables were induced in myogenic, chondrogenic, and pigment cells, and these stained brightly with the anti-F58K. The antibody stained filaments in cultured spinal ganglion cells, as well as the nonneural "background" cells in these cultures. Cultured liver cells, however, were negative.

Immunofluorescent Localization of Anti-BF. Fig. 3 illustrates a cultured chick spinal ganglion cell stained with anti-BF. Fine, long processes stain uniformly, while bright filaments are seen in the perikaryon. The nonneural cells are not stained. The specificity of the anti-BF is greater than the anti-F58K in that it does not bind to the 100-Å filaments in untreated fibroblasts, myoblasts, chondroblasts, or pigment cells. It behaves as nonspecific IgG when applied to CB/Colcemid-treated fibroblasts—i.e., cables are stained at neutral pH, but not when washed at pH 9.5. The anti-BF did not stain the microtubule network in untreated fibroblasts or the mitotic apparatus in cells in metaphase.

Absorption of Anti-F58K and Anti-BF with Purified Smooth Muscle 55,000-Dalton Protein. The staining of several cell types by anti-F58K suggested antigenic overlap among filament subunits, whereas the staining with anti-BF indicated the presence of unique antigenic determinants in neurofilament subunits. Additional evidence for differences between these two antisera was obtained by incubating each IgG with purified insoluble gizzard filament protein. After 18 hr at 4°, the mixtures were centrifuged, and the supernates were tested on untreated and CB/Colcemid-treated fibroblasts and on spinal ganglion cells. Absorbed anti-F58K no longer stained untreated fibroblasts or neurons and reacted as nonspecific IgG on CB/Colcemid-treated fibroblasts. Absorbed anti-BF stained neurons brightly. Absorption of nonspecific IgG with gizzard filament protein had no effect on the nonspecific staining of cables.

Another indication of differences between the 100-Å filaments in fibroblasts and those in nerve cells is obtained from silver staining techniques for visualizing neurofilaments. Normal fibroblasts and fibroblasts with induced cables fail to stain with Protargol or with a modified Bielschowsky silver stain. These procedures impregnated the perikarya and nerve processes of the cultured chick ganglion cells.

Species Specificity of Anti-F58K and Anti-BF. Both anti-F58K and anti-BF produce in mouse and human fibroblasts patterns qualitatively very different from those observed with chick fibroblasts (Fig. 4). The majority of cells exhibit the fluorescent stress fibers conventionally associated with the binding

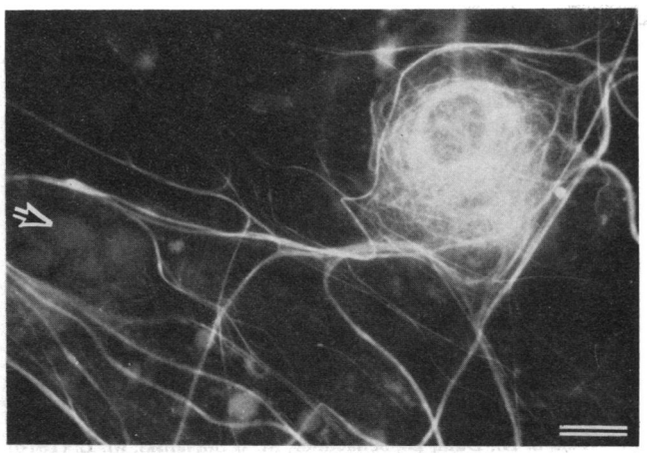

FIG. 3. Chick spinal ganglion neuron, incubated with anti-BF at 1 mg/ml. Note fluorescent processes and filaments within the perikaryon. Several nonneural cells, which do not bind anti-BF, are indicated (arrow). Identical cultures, first extracted with Triton X-100 and then fixed and incubated with anti-BF, give the same pattern. Similar results were also obtained with cultured spinal cord cells. (Bar = 15 μm.)

of anti-actin (29). Occasionally, a periodic anti-myosin-like pattern (30) is also observed. Most significantly, similar patterns are also obtained with many nonspecific IgG fractions. This complex fluorescent pattern is not greatly diminished by washing at pH 9.5.

DISCUSSION

The finding that in fibroblasts the protein subunits of the 100-Å filaments constitute roughly half of the cytoskeleton was unexpected, given our ignorance of their function. The rapid response to Colcemid, particularly in the presence of cycloheximide, azide, cyanide, or deoxyglucose (31), suggests that in many cells either some molecule blocks or its absence precludes spontaneous polymerization and/or assembly into cables. However, until an assay is available for quantitating the number of preexisting 100-Å filaments in untreated cells, it is not possible to determine whether Colcemid (*i*) enhances the polymerization of 58,000-dalton monomers into 100-Å filaments, or (*ii*) only enhances assembly of preexisting filaments into cables. In either case, Colcemid must mimic some function of a normal intracellular metabolite. One possibility is that increasing the local concentration of tubulin subunits blocks an inhibitor of cable formation. Another is that tubulin subunits function as initiation centers for assembly of the cables. It will be interesting to determine whether other native molecules that depolymerize microtubules during mitosis induce the formation of 100-Å filaments.

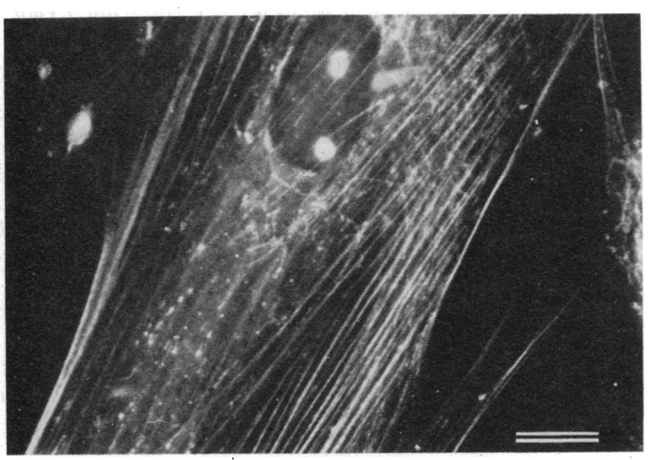

FIG. 4. Human WI 38 fibroblasts incubated with anti-F58K at 1 mg/ml. Similar patterns were obtained with anti-BF and several nonspecific IgGs. Note the conspicuous fluorescence of the stress fibers and the failure of the anti-F58K to bind to any structure having the shape and distribution of 100-Å filaments. (Bar = 15 μm.)

The present data demonstrate antigenic similarities among 100-Å filament subunits from different chick cells, as well as determinants unique to neurofilaments. Anti-BF did not bind to the 100-Å filaments of any cell type other than neurons, and the antibody was not absorbed with purified smooth muscle filament subunits. Anti-F58K exhibited broader specificity, as shown by its binding to the 100-Å filaments of several cell types, including neurons, and by the removal of antibody after absorption with the smooth muscle subunits, despite differences in apparent molecular weight of the fibroblast and smooth muscle proteins. The last observation suggests differences between fibroblast and smooth muscle subunits that are not recognized by our anti-F58K.

More recently, direct evidence for polymorphism has come from comparing the peptide maps of fibroblast, brain, and smooth muscle filament subunits, and with antibodies prepared against gizzard 100-Å filaments. Peptide maps demonstrate differences in primary structure among the subunits from the three sources. The antibody to gizzard 100-Å filaments failed to stain the 100-Å filaments in fibroblasts or nerve cells. Furthermore, the antibody to gizzard filaments formed bands in immunodiffusion tests against gizzard filament subunits, but not against those from fibroblasts or brain (S. A. Fellini, G. S. Bennett, Y. Toyama, and H. Holtzer, unpublished data). Peptide differences were also reported by Davison *et al.* (32) between neurofilament and gizzard filament subunits and by Yen *et al.* (14) between neurofilaments and astrocyte filaments. Studies by Schlaepfer (15) also suggest properties unique to peripheral nerve filaments.

The labeled antibody technique has been a powerful tool for localization of antigens. However, it is sometimes difficult to differentiate spurious binding from antigen–antibody interactions, particularly in the case of structural proteins that have long been known to bind "normal" or "nonspecific" IgG (26–28). The problems are compounded by reports of autoantibodies to "actomyosin" (33), intermediate-sized filaments (20–22), and tubulin (34). One kind of false positive was found in the binding of nonspecific IgG to induced cables. Hynes and Destree (35) noted a similar observation. We found it possible to differentiate the nonspecific binding from antigen–antibody complexes and to show that our anti-F58K and anti-BF do contain antibodies that bind specifically to filamentous structures. The degree of staining of cables by nonspecific IgG appeared to correlate with the diameter of the cables, and could be partly due to trapping of the IgG. These observations require reconsideration of reports of immunological identity of the 100-Å filaments between (*i*) cell types and (*ii*) cells of different species, when induced cables are used as the assay. High concentrations of sera were used in demonstrating that antisera against bovine brain 100-Å neurofilaments bound to induced cables in guinea pig endothelial cells and in chick cardiac cells (16). When we used the same antineurofilament serum used by Blose *et al.* (16) (kindly supplied by M. Shelanski, Harvard University), we found that it failed to stain the 100-Å filaments in normal fibroblasts and that it behaved as a nonspecific IgG on CB/Colcemid-induced cables. Our experience suggests that the following criteria be met before concluding that specific antigen–antibody reactions are responsible for staining 100-Å filaments in immunofluorescence studies: (*i*) Binding of antibodies to aggregates of 100-Å filaments should survive 30–60 min washing at pH 9.5; (*ii*) antibodies should be shown to stain

dispersed filaments in untreated cells. We have routinely used a pH 9.5 wash on both CB/Colcemid-treated and untreated cells. The utility of the high-pH wash to differentiate at least some forms of nonspecific binding of IgG also holds for PtK2 cells. The fluorescence of the tonofilaments in these cells after treatment with many sera (22) is readily abolished after washing at pH 9.5.

It is of interest with respect to species specificity and to the vagaries of the immunofluorescence technique to emphasize what is likely to be another kind of false-positive fluorescence pattern seen in spread mouse and human fibroblasts. These cells have abundant 100-Å filaments and stress fibers identical in distribution to those in chick fibroblasts, as observed under the electron microscope. Nevertheless, the fluorescent staining with anti-F58K is markedly different. Clearly, the conclusion that stress fibers of these cells contain a 58,000-dalton protein is not warranted, given the observation that nonspecific IgG also binds to stress fibers. The nature of the binding to stress fibers is not understood. While we cannot rule out autoantibodies, we consider it equally likely that these findings constitute another instance of nonspecific IgG–macromolecule interaction. Clearly, more extensive studies must be performed before accepting, on the basis of immunofluorescence patterns alone, the presence in stress fibers of any given antigen.

The findings in this report suggest greater polymorphism among the proteins comprising the 100-Å filaments between cell types and species than has been reported for molecules such as tubulin and actin. This degree of polymorphism is more similar to that found among the myosin heavy and light chains (36, 37). At a minimum, there are likely to be five major, overlapping groups: (*i*) the tonofilaments in skin cells, which do not react to Colcemid; (*ii*) the neurofilaments (14, 15, 32); (*iii*) the astrocyte filaments (14, 24); (*iv*) the filaments of the 58,000-dalton protein found in replicating, presumptive myoblasts, fibroblasts, chondroblasts, and pigment cells; and (*v*) the filaments of the 55,000-dalton protein found in smooth muscle cells, postmitotic mononucleated myoblasts and myotubes, cardiac cells, and possibly Schwann cells (38).

Note Added in Proof. Many of our findings complement those reported by Lazarides and Balzer (39), which appeared after this manuscript was submitted for publication.

This research was supported in part by National Institutes of Health Grants HL-18708, HL-15835, CA-18194, and HD-00030; the Muscular Dystrophy Association; and National Science Foundation Grant PCM 77-17930.

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