BIOCHEMICAL AND IMMUNOLOGICAL ANALYSIS OF RAPIDLY PURIFIED 10-nm FILAMENTS FROM BABY HAMSTER KIDNEY (BHK-21) CELLS

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ABSTRACT

Juxtanuclear birefringent caps (FC) containing 10-nm filaments form during the early stages of baby hamster kidney (BHK-21) cell spreading. FC are isolated from spreading cells after replating by treatment with 0.6 M KCl, 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) and DNase I in phosphate-buffered saline. Purified FC are birefringent and retain the pattern of distribution of 10-nm filaments that is seen *in situ*. Up to 90% of the FC protein is resolved as two polypeptides of ~54,000 and 55,000 molecular weight on sodium dodecyl sulfate (SDS) polyacrylamide gels. The protein is immunologically and biochemically distinct from tubulin as determined by indirect immunofluorescence, double immunodiffusion, one-dimensional peptide mapping by limited proteolysis in SDS gels, and amino acid analysis. The BHK-21 FC amino acid composition, however, is very similar to that obtained for 10-nm filament protein derived from other sources including brain and smooth muscle. Partial disassembly of 10-nm filaments has been achieved by treatment of FC with 6 mM sodium-potassium phosphate buffer, pH 7.4. The solubilized components assemble into distinct 10-nm filaments upon the addition of 0.171 M sodium chloride.

KEY WORDS 10-nm filaments · amino acid analysis · indirect immunofluorescence · SDS gel electrophoresis · peptide mapping by limited proteolysis · BHK-21 cells

Intermediate-sized filaments are the least understood of the three types of cytoplasmic fibers present in cells comprising nonmuscle tissues (17, 18, 20). They appear to vary in diameter (7–12 nm) in the various systems in which they have been described. They usually appear as long, flexible rods, which distinguishes them from the larger (~25-nm diam), somewhat rigid microtubule (14, 21, 45) and the thinner (~5–7-nm diam), actin-containing microfilaments (21, 34). The 10-nm, or 100-Å filament, as it is commonly referred to in smooth muscle and cultured cells (7, 18, 36), has also been termed the intermediate filament and neurofilament in cells of neuronal tissues (8, 22, 29, 38, 39, 53) and the tonofilament in cells of epithelial tissues (4, 27, 48). Intermediate filaments appear singly or in bundles along the long axis of axons, and are possibly involved in axonal transport (29). 10-nm filaments form an interconnecting network with dense bodies in chicken gizzards, providing what appears to be a cytoskeletal framework (7). Tonofilaments are present in large numbers in differentiating

epidermis and may represent cytoskeletal elements which insert into desmosomes in epithelial cells (4, 27). They may also function in intracellular organelle transport (16, 20) and locomotion in cultured cells (13, 16, 17).

Filament protein subunits have been termed intermediate filament or neurofilament protein in brain (29, 53), filarin in the squid axon (22), α-keratin (48) and tonofilament protein (5) in epidermis, and skeletin (44), desmin (25, 30) and 100-Å or 10-nm filament protein (7) in smooth muscle. The reported number of polypeptide subunits ranges from 1 to 7 and their molecular weights range from 47,000 to 212,000. It is not known whether these differences are real or artifactual (15). Procedures for isolation of intact filaments or their presumptive subunits are different for different systems and are rather laborious and time-consuming in complex tissues such as brain and smooth muscle (7, 36, 53).

Filaments accumulate in a juxtanuclear position to form a birefringent cap (FC) in spreading baby hamster kidney (BHK-21) cells (19). Large numbers of filaments also aggregate next to the nucleus after treatment with the antimitotic drugs colchicine and vinblastine (1, 4, 16, 20, 23). The apparent increase in the number of filaments seen in these regions as determined by electron microscopy coupled with the breakdown of microtubules has led some researchers to propose a model for the interconversion of microtubules and filaments (33, 52). We and others postulated, however, that only the distribution of filaments was affected by microtubule breakdown (9, 14, 16, 17, 20). In addition, data from several laboratories indicate that filament protein and tubulin differ by various criteria including their immunological properties (26, 42, 53), electrophoretic mobility (8, 40), peptide maps (42, 53), and amino acid composition (8).

We have devised a procedure for obtaining greatly enriched preparations of 10-nm filaments by isolating the birefringent FC which form in colchicine-treated BHK-21 cells (47). An SDS polyacrylamide gel electrophoretic analysis of these preparations demonstrated that ~80% of the protein was resolved in two bands with approximate molecular weights of 54,000 (54K) and 55,000 (55K). Utilizing the same gel system with 6 M urea, α-tubulin was seen to migrate between the two bands of FC protein, and β-tubulin migrated slightly faster than both bands. Roughly 65% of the FC protein isolated from normal, spreading cells was in the same two polypeptides. In this paper, we report a modification of the earlier FC isolation procedure which works equally well on both colchicine-treated and normal, spreading BHK-21 cells. This modified procedure is much faster (~1 h vs. 2 h) and produces preparations in which ~90% of the total protein resides in the 54K and 55K components. The possibility of introducing artifact by using drug-treated cells is now eliminated for the immunological and biochemical analyses described in the present study.

MATERIALS AND METHODS

Cell Cultures

BHK-21/C13[50] cells were grown in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% calf serum, 10% tryptose phosphate broth, and 50 U/ml of penicillin and 50 μg/ml of streptomycin. Mouse neuroblastomas (Nb2a; gift of Dr. Joanna Olmsted) were grown in nutrient mixture F-12 with glutamine (Grand Island Biological Co.) supplemented with 1% fetal calf serum and 50 U/ml of penicillin and 50 μg/ml streptomycin. Stocks of growing cells were maintained at 37°C in plastic tissue-culture dishes (Lux Scientific Inc., Newbury Park, Calif.) kept in a humidified atmosphere of 95% air and 5% CO_2. Frozen stocks of cells were subcultured after removal from the plates with 0.05% trypsin-EDTA solution (Grand Island Biological Co.) into new dishes.

Isolation and Solubilization of BHK-21 FC

Spreading populations of cells containing FC were obtained by trypsinization and replating of confluent cultures onto two-fifths the number of starting dishes. The cells were allowed to spread for 45–60 min. They were then rinsed with two changes of phosphate-buffered saline without added Ca^{2+} or Mg^{2+} (PBSa; 6 mM Na^+-K^+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4), and scraped off the dish with a rubber policeman. All subsequent steps were performed at 4°C and centrifugations were done at 750 *g* for 3 min in a Dynac tabletop centrifuge (Clay-Adams, Div., Parsippany, N. J.). The cells were collected by centrifugation, and the pellet was quickly resuspended in 2.5 ml of lysing solution/100 mm dish. The lysing solution consisted of 0.6 M KCl, 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.)/PBSa with the protease inhibitors 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, Mo.) and 1 mg/ml *p*-tosyl-L-arginine methylester-HCl (TAME, Sigma Chemical Co.). The suspension became very viscous as the chromatin was released from the lysed nuclei. $MgCl_2$ and DNase I (Sigma Chemical Co.) were added up to final concentrations of 10 mM and 0.3–0.5 mg/ml, respectively. The viscosity dropped

sharply within minutes, and the birefringent FC were pelleted by centrifugation. The pellet was washed four times in PBSa containing 5 mM EDTA, to remove the excess salts and remaining detergent. Solubilization of BHK-21 FC was achieved after two washes with 6 mM Na^+-K^+ phosphate buffer, pH 7.4, 0.1 mM PMSF, and 1 mg/ml of TAME. The final pellet was resuspended in a small volume of the same buffer, giving a final protein concentration of 2–3 mg/ml. Protein concentration was determined by the micro-biuret method (24).

Preparation of Tubulin

Chicken brain tubulin was purified with three assembly-disassembly cycles (41). BHK-21 tubulin was obtained from vinblastine (VB)-induced paracrystals prepared by modifying the technique described by Nagayama and Dales (31). VB-treated cells (10 μg/ml, 48–72 h) were removed from the dish with a rubber policeman in growth medium containing VB. Cells were pelleted by centrifugation at 370 *g* for 5 min. The pellet was homogenized in 1% Nonidet P-40 (Shell Chemical Co., New York), in PBSa with VB, and then sheared by forcing through a 26-gauge needle. The suspension was mixed with an equal volume of Genesolv D (Allied Chemical Corp., Morristown, N. J.) and centrifuged at 190 *g* for 5 min. The upper phase was centrifuged at 1,900 *g* for 20 min, and the pellet was resuspended in PBSa with VB and re-extracted twice with an equal volume of Genesolv D. Higher yields of crystals could be obtained by mixing the first Genesolv D phase with an equal volume of PBSa with VB, and centrifuging at 190 *g* for 5 min. Crystals were collected from the upper phase by centrifuging for 20 min at 1,900 *g* and pooled with the original fraction.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

Electrophoretic analysis of FC protein and tubulin was carried out in 7.5% SDS polyacrylamide vertical slab gels (28) with 4.5% acrylamide stacking gels. Constant current (25 mA) was applied for 2½–3 h and turned off when the tracking dye (Pyronin Y; Allied Chemical Corp., Morristown, N. J.) reached ~5 mm from the bottom of the gel. Gels were stained for 2 h with 0.1% Coomiassie Blue R (ICI United States Inc., Wilmington, Del.), 50% methanol, and 10% acetic acid, and destained by diffusion in 10% acetic acid.

One-Dimensional Peptide Mapping by Limited Proteolysis

Protein bands for the proteolysis procedure (6) were sliced from gels prepared as described above. However, staining was allowed to proceed for only 20 min to visualize the bands, and no destaining was necessary. The slices were stored overnight at −20°C in 0.125 M Tris/HCl, pH 6.8, 0.1 % SDS, and 1 mM EDTA. Proteolytic digestion in gel slices was performed exactly as described by Cleveland et al. (6) with the protease *Staphylococcus aureus* V8 (Miles Laboratories Inc., Elkhart, Ind.).

Amino Acid Analyses

Amino acid analyses were performed on both a Beckman 120C Amino Acid Analyzer (Beckman Spinco, Palo Alto, Calif.) and a Durrum D-500 autoanalyzer (Durrum Instrument, Sunnyvale, Calif.). BHK-21 FC were hydrolyzed for 24, 48, and 72 h in 6 N HCl under vacuum at 110°C. Cysteine was quantitated as cysteic acid according to the procedure of Spencer and Wold (46).

Light Microscopy

Light micrographs were taken on a Zeiss Photomicroscope III equipped with either polarization or epifluorescence optics. Polarized light micrographs were taken on Kodak Panatomic-X film utilizing a 100-W tungsten source. Fluorescence micrographs were taken with Kodak Plus-X film utilizing a xenon lamp.

Electron Microscopy

Cells were fixed in 1% glutaraldehyde in 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in PBSa (PBS) for 30 min, washed in PBS, postfixed in 1% OsO_4 in PBS for 30 min, and dehydrated in 70, 95, and 100% ethanols. The cells remained in a 1:1 mixture of Epon-Araldite (51): absolute ethanol overnight, and were embedded in Epon-Araldite the following day. Isolated BHK-21 FC were fixed and embedded as pellets in capsules.

Thin sections were made on an LKB Ultrotome III (LKB Instruments, Rockville, Md.), mounted on uncoated grids, and stained with 3% uranyl acetate for 10 min, followed by Reynolds' lead citrate (35) for 10 min. Negative stains were made on Formvar carbon-coated grids with 1% uranyl acetate. Electron micrographs were taken on a Philips 201C electron microscope.

Preparation of BHK-21 FC Antiserum and Immunological Analyses

Two 1-kg New Zealand rabbits were injected in one footpad and subcutaneously into the neck with 1.6 mg of BHK-21 FC protein homogenized in 0.5 ml of 10 mM Tris/HCl, pH 7.5, and emulsified with an equal volume of Freund's complete adjuvant. The rabbits were boosted twice intramuscularly at 21 and 26 days after the initial injection, with 0.6 mg of antigen without adjuvant. The rabbits were bled 1 wk after the last booster by ear puncture, and terminally by cardiac puncture in the subsequent week. The blood was allowed to coagulate for 2 h at 37°C, and overnight at 4°C. Serum was obtained by two cycles of centrifugation at 1,900 *g* for 10 min (4°C). The sera were aliquoted into small tubes and stored at −20°C. Preimmune serum was

collected before initial injection by cardiac puncture.

Ouchterlony's double immunodiffusion analyses (32) were performed in 60-mm plastic dishes containing 1% purified Difco agar (Difco Laboratories, Detroit, Mich.) in 0.6 M KCl, 10 mM Tris/HCl, pH 7.5, at 4°C for 72–96 h. Dishes were washed for 36 h in 0.6 M KCl, 10 mM Tris/HCl, pH 7.5, and for 36 h in glass-distilled water before staining in 0.25% Coomassie Blue R, 50% methanol, and 10% acetic acid for 20 min. The agar plates were destained by diffusion in 20% methanol-10% acetic acid, followed by 10% acetic acid. One of the two rabbits produced an antibody as determined by this method.

Indirect immunofluorescence was performed on cells grown on glass cover slips that were acetone-fixed at −20°C for 1 min. The cover slips were allowed to air-dry before control or immune serum was layered on top. The cover slips were incubated in a humidified atmosphere at 37°C for 30 min-1 h and washed thoroughly in PBS. They were then incubated with fluorescein-labeled goat anti-rabbit IgG (Miles-Yeda Research Development Co., Ltd., Rehóvoth, Israel; purified by ion-exchange chromatography) for 30 min-1 h at 37°C, washed thoroughly in several changes of glass distilled water, and mounted on glass slides for observation (16).

RESULTS

Isolation of BHK-21 Filament Caps

BHK-21 cells, as they appeared in the polarizing microscope during the early stages of spreading, are shown in Fig. 1 *a* and *b*. The birefringent sphere or cap, discussed in detail elsewhere (19), has been shown to consist almost exclusively of 10-nm filaments (Fig. 1*c*). Enriched preparations of filaments were routinely obtained in 45–60 min from spreading populations of cells, in the form of FC. The remainder of the cell contents were effectively dispersed and/or solubilized during a one-step treatment with 0.6 M KCl, 1% Triton X-100/PBSa, followed by DNase I. Purified FC maintained their birefringence (Fig. 2*a* and *b*), and the filaments were morphologically identical to those seen *in situ* (compare Figs. 2*c* and 1*c*). In addition to large numbers of 10-nm filaments seen in thin sections of FC, paired centrioles were occasionally seen as well as a small amount of amorphous material (Fig. 3). Whether the co-purification of centrioles and 10-nm filaments reflects an in vivo association or is simply fortuitous is not known at the present time.

Two major bands of protein were resolved on SDS gels of BHK-21 FC corresponding to molecular weights of ~54K and ~55K (47). FC prepared by the technique presently described contained up to 90% of the total protein, as determined by densitometric tracings of SDS gels (not shown), in the two major bands (Fig. 7*c*). The most prominent of the minor components appeared to be a polypeptide between 250,000 and 350,000 molecular weight. Approximately 170 μg of FC protein could be obtained from one dish of spreading cells.

Immunological Analysis

An antiserum to BHK-21 FC was prepared that specifically interacts with a component of 10-nm filaments. Studies using indirect immunofluorescence on spreading BHK-21 fibroblasts revealed fluorescence in areas known to contain 10-nm filaments by electron microscopy (16, 17, 19, 20). Bright juxtanuclear circular regions were observed in the earlier stages of spreading before the appearance of bright fibers as the cell flattened on the substrate (Fig. 4). These observations were similar to those described above for living cells (cf. Figs. 1 and 4). This antiserum also reacted with cytoplasmic regions enriched for 10-nm filaments in Nb2a cells (Fig. 5).

The immune serum was also used to demonstrate that 10-nm filament protein was immunologically distinct from tubulin from the same cell line. BHK-21 cells, treated with VB (10 μg/ml), formed both birefringent tubulin-containing paracrystals and 10-nm filament caps (Fig. 6*a* and *e*). Cells prepared for indirect immunofluorescence, as shown in Fig. 6*b–d*, contained fluorescent FC adjacent to dark paracrystals. Partially purified preparations of paracrystals which were greatly enriched for α- and β-tubulin (Figs. 6*f* and 8*b*) were used to further test the lack of cross-reaction by Ouchterlony's double immunodiffusion analysis (32) (Fig. 7). No precipitin line was detectable with tubulin from BHK cells or chicken brain. However, a single precipitin line was observed between FC from both colchicine-treated (47) and spreading BHK-21 cells. The preimmune serum, used as a control, neither stained cells (Fig. 4*d*) nor formed a precipitin line with the antigens tested.

Peptide Mapping by Limited Proteolysis

The technique of Cleveland et al. (6) for one-dimensional peptide mapping by limited proteolysis in SDS gels was used to determine whether any similarities existed between the 54K and 55K components of BHK-21 FC, and to compare these

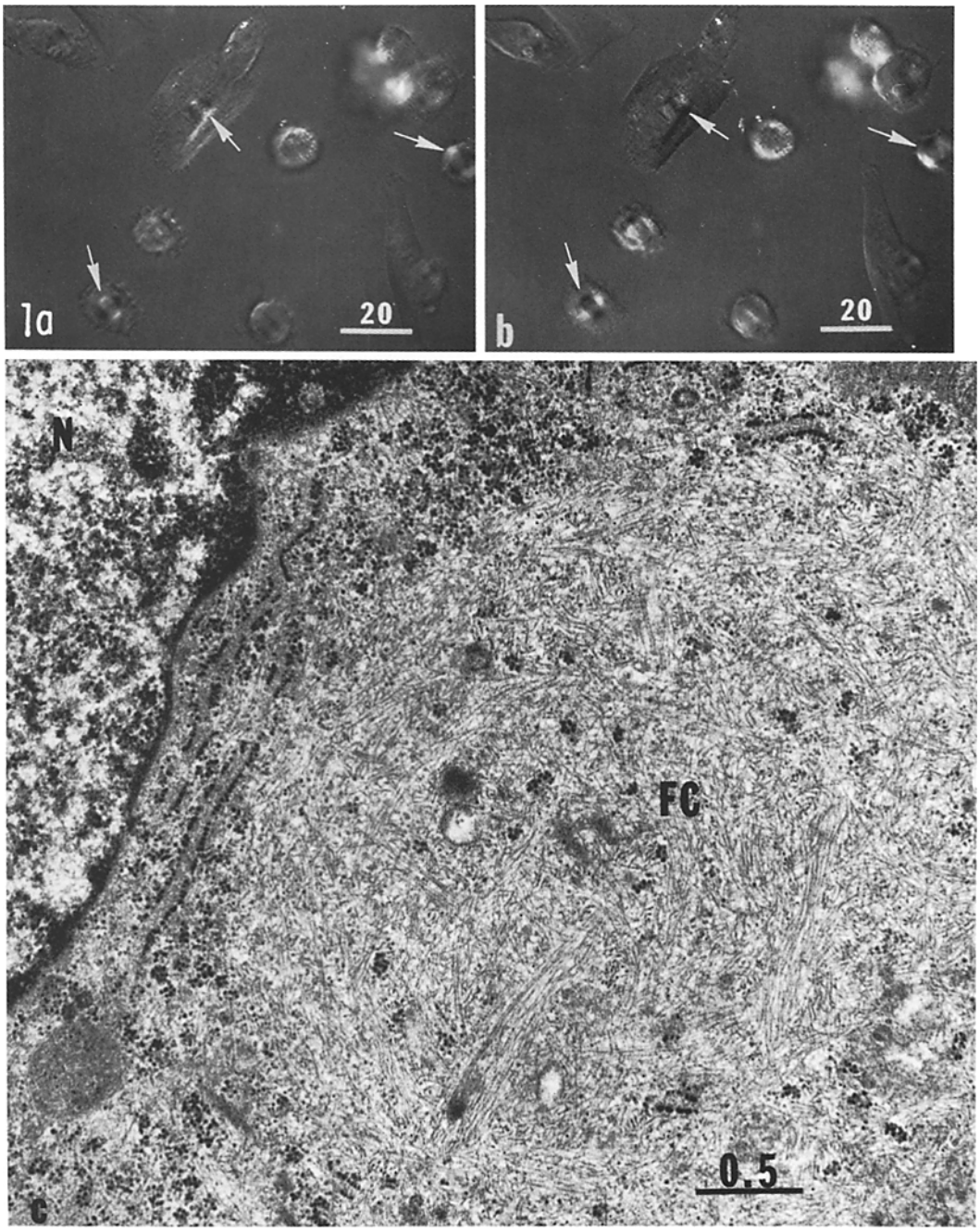

FIGURE 1 (*a* and *b*) Living BHK-21 cells 2 h after being replated onto a glass cover slip. Many birefringent FC are apparent (arrows). Polarized light with a Zeiss Brace-Koehler mica compensator (λ/30), at opposite settings. × 500. (*c*) Thin section through a spreading cell showing a juxtanuclear accumulation of 9–10-nm filaments. *N*, nucleus. × 30,000. The scale is given in microns in this figure and in all other figures.

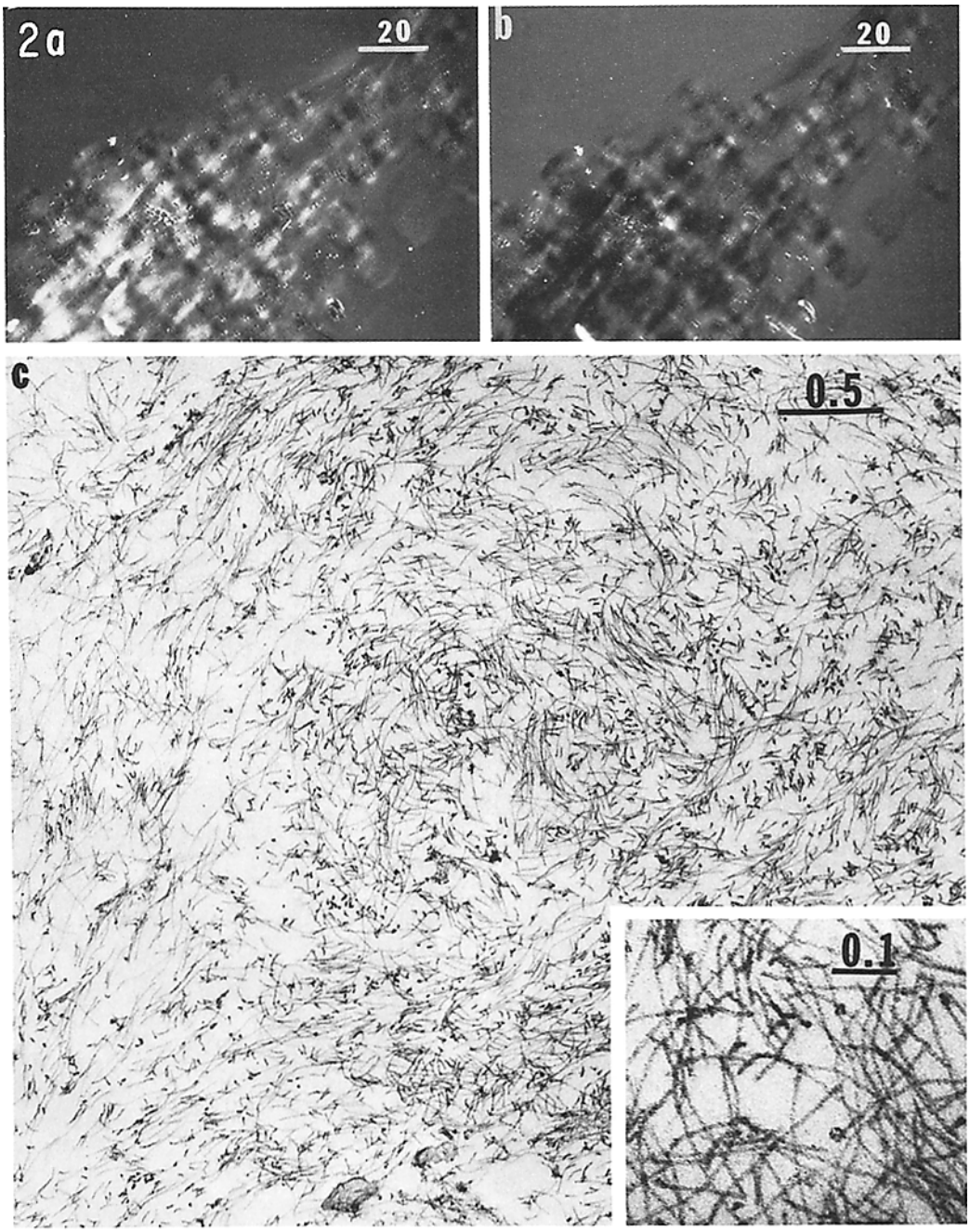

FIGURE 2 (*a* and *b*) Purified FC from spreading BHK-21 cells. Polarized light at opposite compensator settings. × 500. (*c*) Thin section through a pellet of isolated FC which is seen to contain large numbers of 9–10-nm filaments. × 30,000. Insert, × 90,000.

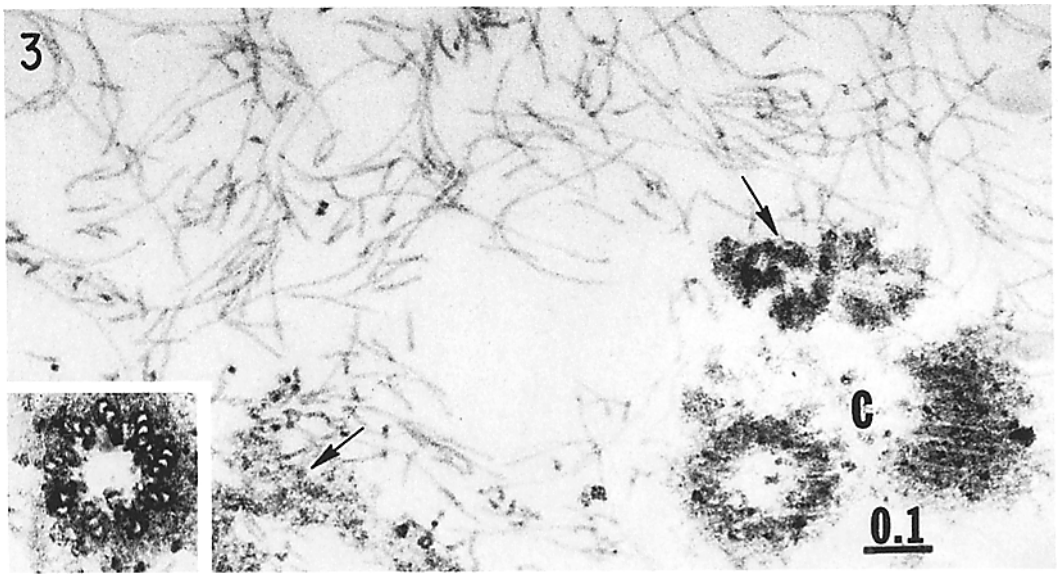

FIGURE 3 A thin section revealing a pair of centrioles (*C*) and some amorphous material (arrows) surrounded by filaments in an FC preparation. × 90,000. Insert: a cross section of a centriole in another FC preparation in which the typical 9+0 arrangement of triplet microtubules is apparent. × 90,000.

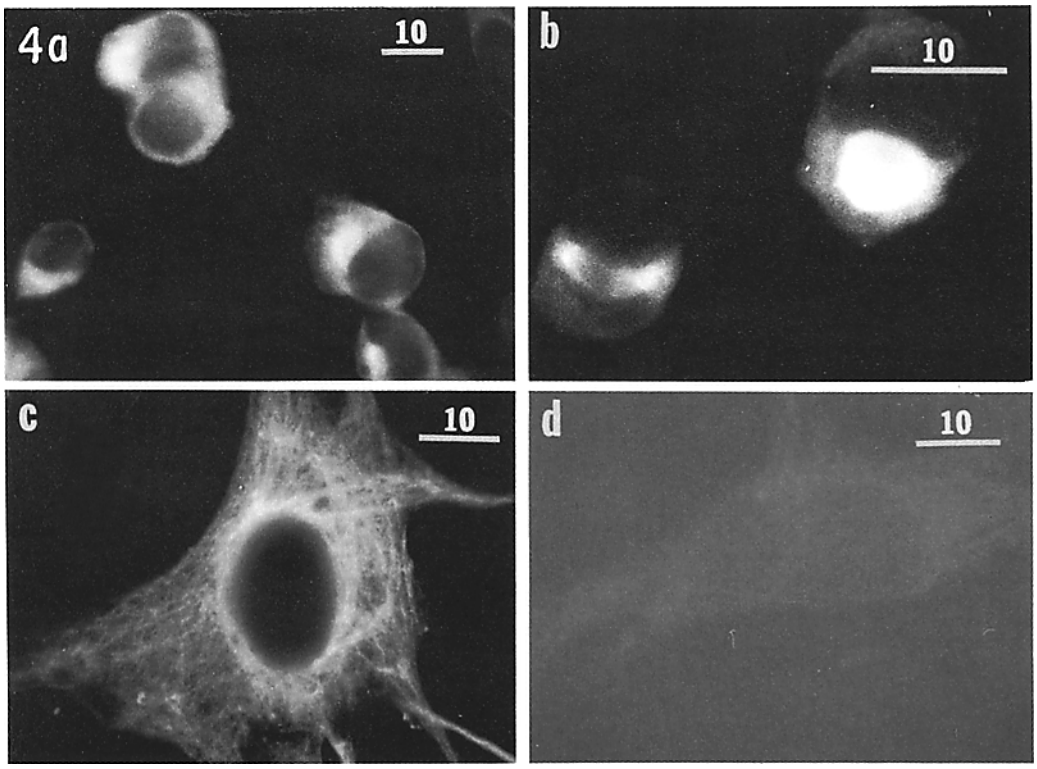

FIGURE 4 Indirect immunofluorescence of BHK-21 cells, fixed and stained, as described in Materials and Methods, with a 1:30 dilution in PBS of immune and control sera. Fluorescein-labeled goat anti-rabbit IgG was used at a concentration of 0.5 mg/ml. (*a* and *b*) Spreading cells containing fluorescent FC 1½ h after replating onto glass cover slips. Epifluorescence, (*a*) × 800, (*b*) × 1,800. (*c*) A well-spread cell in which a fibrous network is seen to originate from a juxtanuclear position. Epifluorescence, × 1,100. (*d*) Preimmune serum control. Epifluorescence, × 1,100.

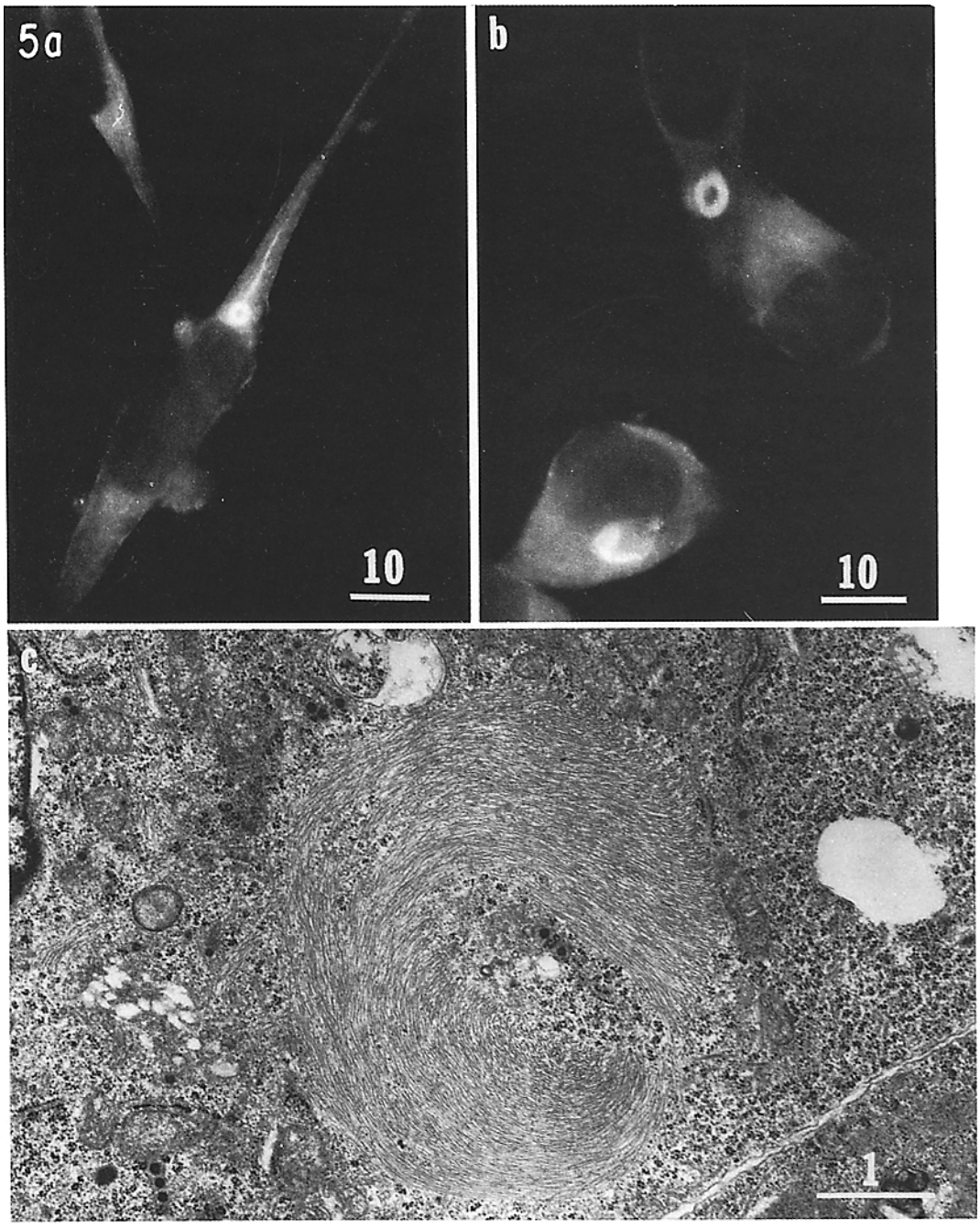

FIGURE 5 (*a* and *b*) Mouse neuroblastoma cells prepared for indirect immunofluorescence with BHK-21 FC antiserum reveal bright cytoplasmic structures that correspond to (*c*) aggregates of 10-nm filaments. Epifluorescence, (*a*) × 1,100, (*b*) × 1,200. (*c*) Thin section, × 17,000.

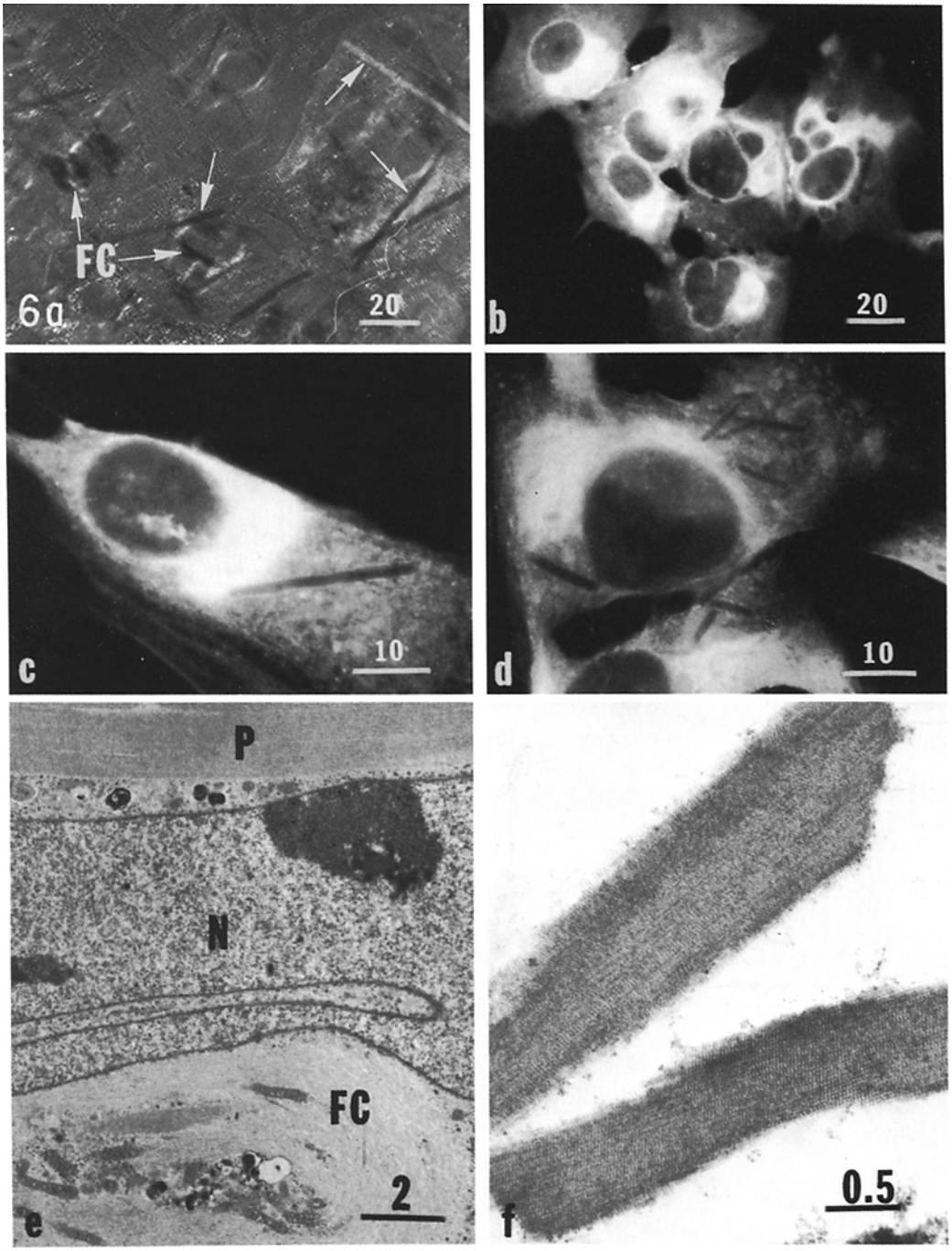

FIGURE 6 (*a*) Living cells treated with VB (10 μg/ml) for 48 h. Both birefringent crystals (arrows) and FC can be seen. Polarized light, × 450. (*b*) VB-treated cells prepared for indirect immunofluorescence to visualize FC. Epifluorescence, × 450. (*c* and *d*) Same as *b*. Note the dark tubulin paracrystals. × 1,100. (*e*) Thin section of a VB-treated cell showing a paracrystal (*P*) and FC. *N*, nucleus. × 6,000. (*f*) Thin section of a pellet of partially purified paracrystals. × 20,000.

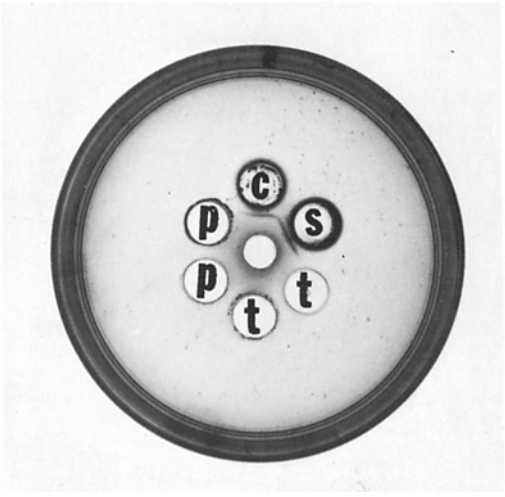

FIGURE 7 Double immunodiffusion analysis of BHK-21 antiserum (center well) with colchicine-induced FC (*c*), and FC from spreading cells (*s*), both in 8 M urea, 10 mM Tris/HCl, pH 7.5. BHK tubulin (*p*) and chicken brain tubulin (*t*) at two different concentrations were in 10 mM Tris/HCl, pH 7.5. A reaction is seen between the antiserum and FC from normal and drug-treated cells, but not with either of the tubulins.

polypeptides with α- and β-tubulin. Fig. 8*a–c* shows a 7.5% SDS polyacrylamide gel (28) containing FC protein, an enriched preparation of BHK-21 vinblastine paracrystals, and chicken brain tubulin. Bands were cut out from a similar gel for peptide mapping (Fig. 8*d–j*) and for electrophoresis on a 10% SDS polyacrylamide-8 M urea gel (Fig. 8*k* and *l*). Some degree of homology was seen in the patterns generated from the 54K and 55K components (Fig. 8*e* and *f*), so the urea-SDS system, which maximizes the separation of the 54K and 55K components (47), was utilized

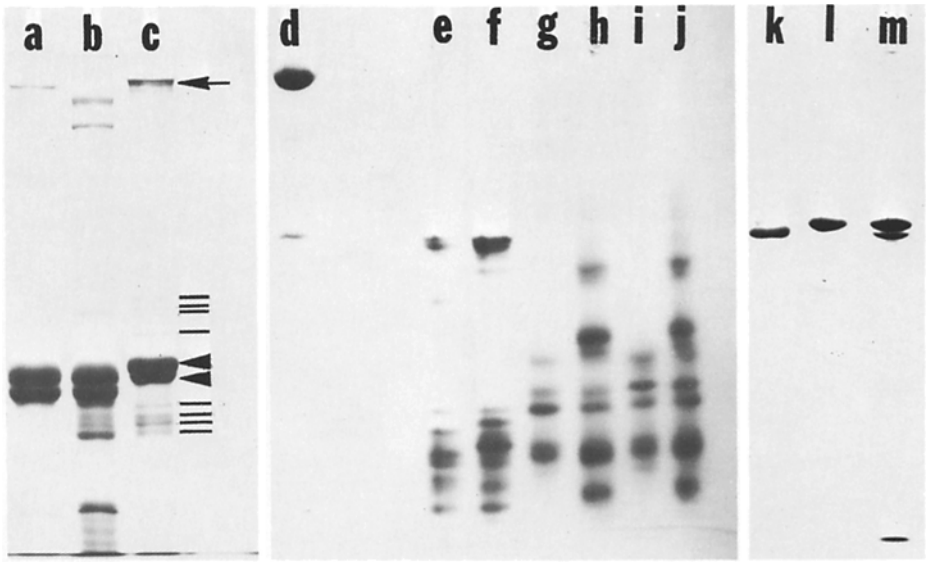

FIGURE 8 (*a–c*) A 1-mm thick 7.5% SDS polyacrylamide gel (28) containing (*a*) 10 μg of chicken brain tubulin, (*b*) 15 μg of BHK paracrystals, and (*c*) 10 μg of BHK-21 FC protein. The arrow indicates the high molecular weight protein, and the arrowheads indicate the 54K and 55K components. The minor components are marked with lines. (*d–j*) One-dimensional peptide mapping by limited proteolysis (6) in a 12.5% SDS polyacrylamide gel with a 3.5-cm stacking gel. No protease was added to *d* where a gel slice containing ~15 μg of the BHK-21 FC 54K and 55K polypeptides was placed. However, some breakdown has occurred. This is probably due to some protease leaking over from nearby wells, since only a single band was seen when the protein was run on a 12.5% gel as described (6) with the exception that no protease was added to any of the wells. 0.25 μg of protease *S. aureus* V8 was layered on the gel slices in wells *e–j*. (*e*) 55K protein, (*f*) 54K protein, (*g*) BHK α-tubulin, (*h*) BHK β-tubulin, (*i*) chicken brain α-tubulin, (*j*) chicken brain β-tubulin, (*k–m*) a 10% SDS polyacrylamide-8 M urea gel, (*k*) a gel slice containing 54K protein, (*l*) a gel slice containing 55K protein, (*m*) 5 μg of BHK-21 FC protein. Minor components (see Fig. 8*c*) are not easily seen at this protein loading.

to determine whether cross-contamination occurred when the bands were sliced from the gel. A very small amount of 55K protein could occasionally be seen in the 54K gel slice but it is doubtful that the protein, once digested, could contribute very much to the peptide maps. Until further data are obtained, the available evidence indicates that the 54K and 55K components represent similar polypeptides.

The digestion pattern of BHK α- and β-tubulin, shown in Fig. 8*g* and *h*, was distinctly different from that obtained from the 54K and 55K proteins of BHK 10-nm filaments. The patterns of the digests of α- and β-tubulin from BHK-21 (Fig. 8*g* and *h*) and chicken brain (Fig. 8*i* and *j*) are similar to the patterns obtained by Cleveland et al. (6) using the same protease (*S. aureus* V8).

Amino Acid Analyses

The amino acid analysis of BHK-21 FC is shown in Table I (column 8). The compositions of 10-nm filament proteins from a variety of sources are included in the table for comparison (columns 1–6). Considerable homology exists between the BHK-21 FC and the other filament proteins as indicated by the fact that 15 of the 17 amino acids analyzed for BHK-21 FC fall within the range of values (column 7) determined for the other filament proteins. Furthermore, the remaining two amino acids are close to the indicated ranges. In contrast, the dissimilarity of 10-nm filament protein and tubulin is emphasized in Table II, where the compositions of 10-nm filament and a composite range of values for the composition of tubulin are compared. Here, 10 of the 17 amino acids analyzed for BHK-21 FC fall outside the range of values determined for tubulin.

10-nm Filament Disassembly-Reassembly

Efforts to study the disassembly and reassembly

TABLE I
Amino Acid Analysis of 10-nm Filament Protein

	Human astrocytes* (11)	Hog stomach‡ (44)	Chicken gizzard‡ (44)	Chicken gizzard§	Bovine epidermis‖ (48)	Bovine brain¶ (8)	Range	BHK-21 FC**
Asp	9.1	9.2	8.8	9.5	9.7	9.5	8.8–9.7	10.3 (0.5)
Thr	4.7	5.2	5.7	5.7	3.9	4.8	3.9–5.7	4.8 (0.6)
Ser	5.9	7.6	7.4	7.5	10.5	3.4	3.4–10.5	6.5 (1.8)
Glu	18.4	19.5	19.7	13.5	14.8	16.6	13.5–19.7	18.7 (1.5)
Pro	3.0	2.9	2.3	3.8	1.3	0.0	0–3.8	2.9 (0.2)
Gly	4.9	5.1	5.0	11.0	12.9	6.0	4.9–12.9	5.7 (0.9)
Ala	10.2	9.0	8.6	9.9	6.6	9.8	6.6–10.2	8.4 (0.4)
Val	4.5	4.9	5.0	5.4	5.7	4.5	4.5–5.7	5.4 (0.3)
Cys	0.0	0.7	0.5	0.9	0.9	0.0	0.0–0.9	0.8
Met	2.0	2.0	2.5	2.2	2.0	2.1	2.0–2.5	2.0 (0.1)
Ile	3.2	3.9	4.2	4.3	3.8	3.9	3.2–4.3	3.8 (0.1)
Leu	11.8	10.0	9.9	8.1	10.2	11.6	8.1–11.8	11.2 (0.9)
Tyr	2.8	2.8	3.0	2.2	2.4	4.1	2.2–4.1	2.3 (0.3)
Phe	2.7	2.7	2.7	3.3	3.7	3.8	2.7–3.8	2.3 (0.2)
Lys	6.0	4.7	4.7	5.3	4.6	5.7	4.6–6.0	5.5 (0.7)
His	1.9	1.3	1.6	1.5	1.2	2.0	1.2–2.0	1.5 (0.1)
Arg	8.5	8.6	8.7	5.9	6.5	9.7	5.9–9.7	8.1 (0.4)

* Data expressed in mole percent for electrophoretically purified protein subunits.

‡ Expression of data unclear.

§ Data expressed in residues per 100 residues for electrophoretically purified protein subunits. (P. Cooke, Department of Physiology, School of Medicine, The University of Connecticut, Farmington, Conn. Personal communication).

‖ Data expressed in residues/100 residues for the electrophoretically purified major component (polypeptide 5).

¶ Data expressed in moles per 100 residues.

** These data (expressed in mole percent) are the means of four independent analyses after 24-h hydrolysis of three different preparations of BHK-21 FC. Numbers in parentheses represent the standard deviation. Cysteine was determined in a single analysis as cysteic acid after hydrolysis in the presence of DMSO (46). Threonine and serine values have been corrected for decomposition, using factors derived from extrapolations of 24-, 48- and 72-h hydrolysis to zero time.

TABLE II

Amino Acid Composition of BHK-21 FC and Tubulin

	BHK-21 FC	Tubulins (49)
	mol/10⁵ g of protein	*mol/10⁵ g of protein*
Asp	109.5	85–94
Thr	51.6	46–61
Ser	68.9	45–59
Glu	199.7	104–124
Pro	31.2	39–46
Gly	61.0	60–80
Ala	89.5	56–71
Val	57.5	51–58
Cys	8.8	6–25
Met	21.3	20–32
Ile	40.2	39–49
Leu	119.9	63–76
Tyr	25.1	25–33
Phe	24.7	33–41
Lys	58.7	34–56
His	16.4	20–25
Arg	86.3	38–49

of 10-nm filaments obtained from BHK-21 cells were undertaken to ascertain which subunits might be utilized to assemble filaments in vitro. Soluble fractions of 10-nm filament protein were obtained from intact filament caps after room temperature treatment for 2 h with 6 mM Na^+-K^+ phosphate buffer, pH 7.4, containing the protease inhibitors PMSF (0.1 mM) and TAME (1 mg/ml). Fig. 9 shows the effects of this buffer on a clump of purified birefringent FC that was lodged between a cover slip and slide. Birefringence disappeared rapidly and was followed by dispersal of the cap structures. However, intact 10-nm filaments were present when these preparations were observed by negative staining (Fig. 10*a*). The suspensions were then centrifuged at 60,000 *g* for 1 h in an attempt to remove intact filaments. 60–80% of the protein remained in the supernate, but very few intact filaments were seen after this centrifugation (Fig. 10*b*). A layer of white lipid-like material, found on the surface, was removed with a Pasteur pipette. When NaCl was added to this solution to equal the concentration present in PBSa (0.17 M), viscosity and turbidity increases were noted within minutes (at room temperature). Negative stains revealed large numbers of filaments about 10 nm in diameter which formed within and between amorphous clumps of material. The filaments appeared to grow longer with time (Fig. 10*c* and *d*). After centrifugation again at 60,000 *g* for 1 h, a small clear pellet of filaments was obtained (Fig. 11) that contained both the 54K and 55K components and the high molecular weight protein as determined by SDS

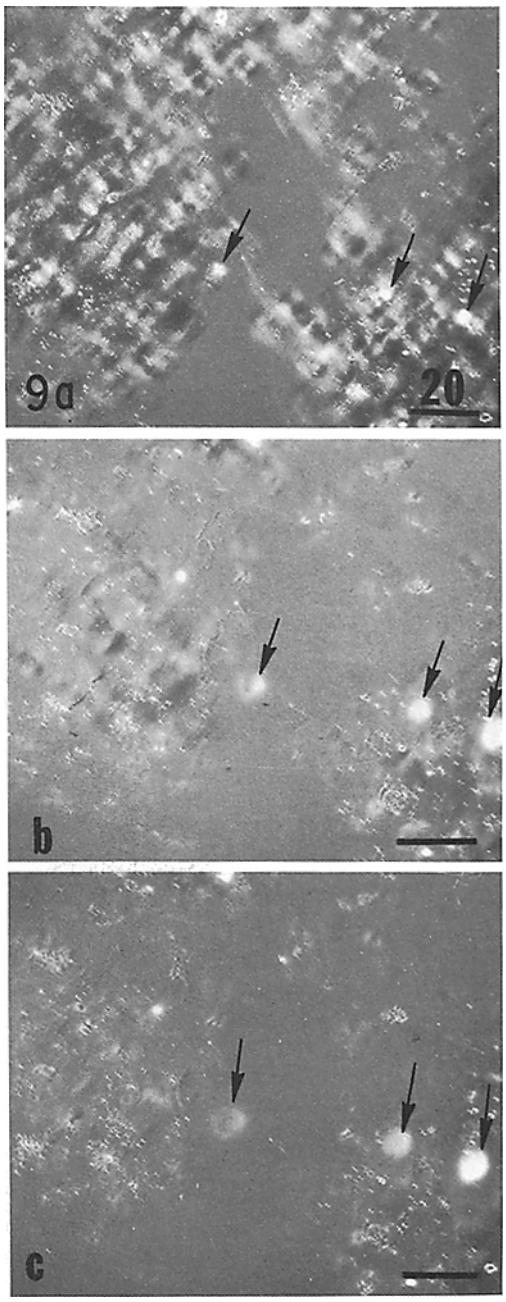

FIGURE 9 Effects of 6 mM Na^+-K^+ phosphate, pH 7.4, on BHK-21 FC. (*a*) FC in PBSa. (*b*) 5 min after the phosphate buffer was perfused under the coverslip. (*c*) 10 min later. Arrows mark refractile structures in the microscope field for purpose of orientation. Polarized light, × 500.

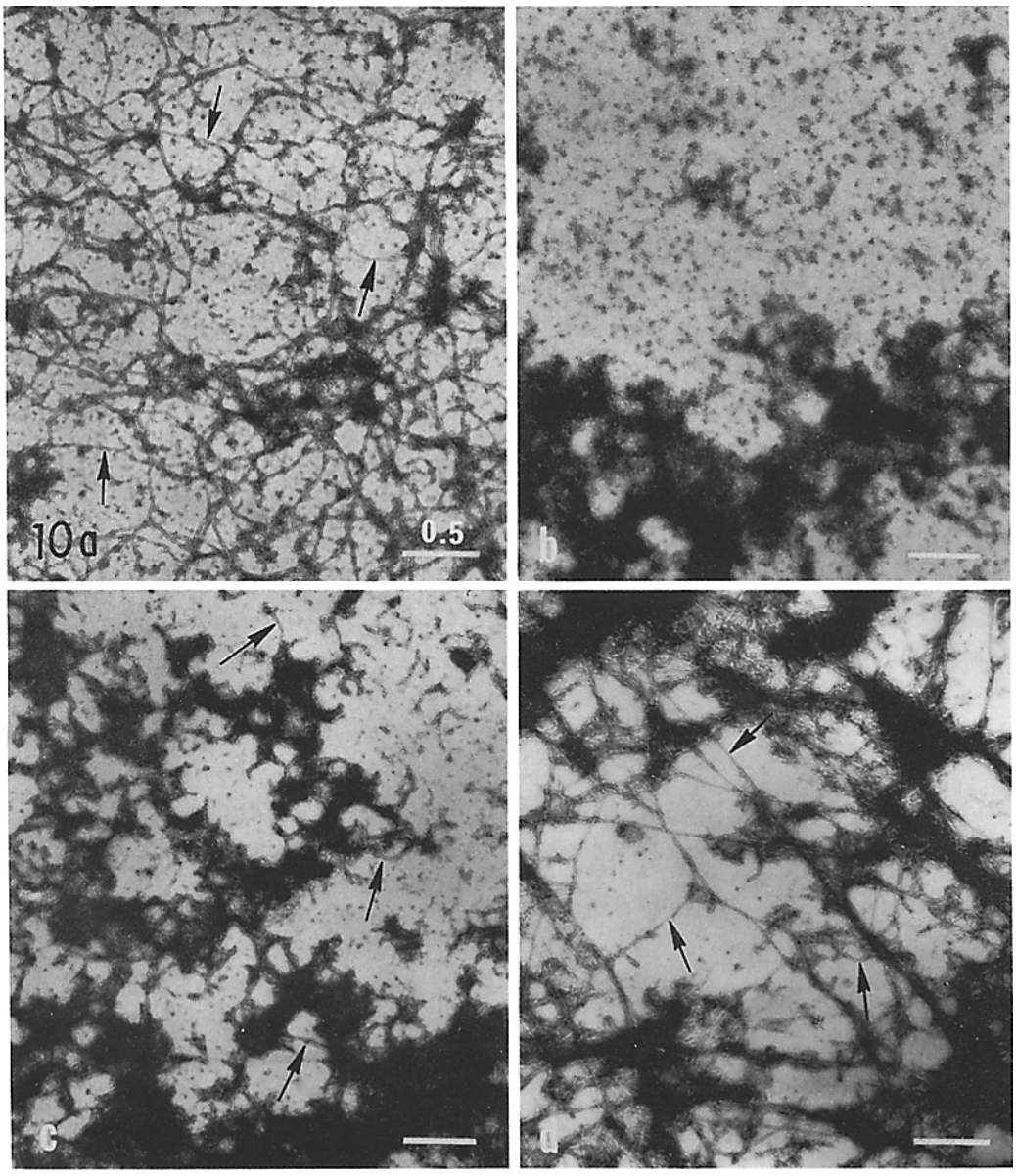

FIGURE 10 Negative staining of (*a*) remaining 10-nm filaments (arrows) after treatment with 6 mM Na^+-K^+ phosphate buffer. (*b*) Supernate after centrifugation at 60,000 *g* for 1 h. A large amount of amorphous material but few intact filaments are seen. (*c*) 2 h after the addition of NaCl (0.17 M). 10–12-nm filaments (arrows) are forming between and within the amorphous clumps. (*d*) 24 h after the addition of NaCl. A network of tangled, long filaments (arrows) is seen that closely resembles freshly isolated filaments. × 20,000.

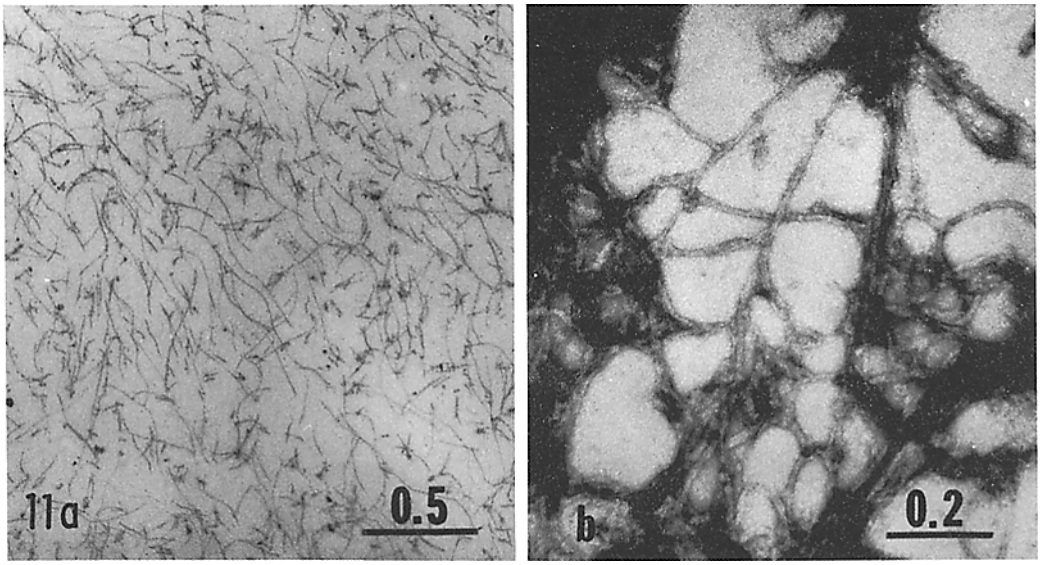

FIGURE 11 (*a*) Thin section and (*b*) negative staining of a pellet of reassembled filaments. (*a*) × 29,000. (*b*) × 67,000.

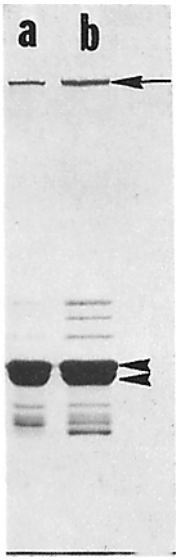

FIGURE 12 A 7.5% SDS polyacrylamide gel (28). (*a*) Reassembled filaments pelleted by centrifugation at 60,000 *g* (10 μg). (*b*) The 60,000-*g* pellet obtained after incubation with phosphate buffer (10 μg). Arrow, high molecular weight protein. Arrowheads, 54K and 55K proteins.

gel electrophoresis (Fig. 12). It was also found that the BHK-21 FC antiserum formed a precipitin line with the original 60,000-*g* supernate when assayed by double immunodiffusion.

DISCUSSION

BHK-21 10-nm Filament Caps

Highly enriched preparations of 10-nm filaments can be obtained rapidly and easily from normal BHK-21 cells. The observation of paired centrioles in the pellet of purified FC is not surprising since electron microscopists have observed filament-centriole associations in other cell types *in situ* (37). Therefore, it is possible that some of the minor protein components present in the FC preparations may originate from the centrioles. We are quite certain that the major bands seen on SDS gels are components of 10-nm filaments since the number of centrioles relative to filaments seen in thin sections of FC is very small. In addition, centrioles are present in the pelleted fraction after treatment with 6-mM Na^+-K^+ phosphate and cannot be found in the reassembled filament preparations which contain the major 54K and 55K subunits, or in FC from colchicine-treated BHK-21 cells prepared as previously described (47).

Peptide Mapping by Limited Proteolysis

The elegant technique of Cleveland et al. (6) for one-dimensional peptide mapping has proved to be very reliable and reproducible in our laboratory. Since it has been proposed that tubulin and 10-nm filaments might be composed of the same or similar proteins (33, 52) and that both

proteins comigrate on SDS gels (47) prepared according to Fairbanks et al. (12), tubulin and FC protein were compared by the limited proteolysis procedure. The major polypeptides released from both the 54K and 55K components are distinctly different from tubulin from the same cell line and from chicken brain.

Similarities among 10-nm Filament Proteins

The amino acid composition of BHK-21 FC is very similar to the compositions of filament protein from chicken gizzard (P. Cooke, Department of Physiology, School of Medicine, The University of Connecticut, Farmington, Conn. Personal communication.) (44), hog stomach (44), human astrocytes (11), bovine brain (8), and epidermis (48). Another indication that there is homology between 10-nm filaments from different sources is provided by the observation that BHK-21 FC antiserum cross-reacts with Nb2a cells, as determined by indirect immunofluorescence. These data agree with the work of others who have suggested that the major protein subunits from different species and cell types may be essentially the same (2, 14, 44).

Disassembly-Reassembly

The study of the disassembly and reassembly of 10-nm filaments has physiological significance since the process appears to be an integral component of the BHK-21 cell cycle (10, 17). During metaphase, very few 10-nm filaments are seen in thin section. However, shortly after cytokinesis, birefringent FC are seen in the spreading daughter cells, corresponding to large accumulations of filaments (17). A soluble fraction of 10-nm filament protein has been obtained that will form filaments under physiological conditions. This finding can be used to support data from other laboratories which indicate that filament protein may exist in nonfilamentous forms such as the soluble glial fibrillary acid protein of brain (14), and as a component of the Z lines of skeletal muscle (30). Work by other laboratories has involved solubilization with 4 M guanidine hydrochloride (8), 8 M urea (7), or 1 M acetic acid (44), and reassembly upon removal of these agents by dialysis.

A protein of between 250,000 and 350,000 molecular weight, present in freshly isolated BHK-21 FC, is also seen in the 60,000-*g* supernate and is pelleted with the reassembled filaments after addition of NaCl. Efforts are underway to determine whether or not it is an integral component of the filaments. The high molecular weight proteins that co-purify with microtubule protein (MAPS or HMWS) have been shown to initiate assembly, form fuzzy-walled microtubules, and have a slight ATPase activity (3, 43). A microtubule-10-nm filament complex has been described in BHK-21 cells, and fine fibrillar material appears to form cross bridges between microtubules and surrounding filaments (see Fig. 7 in reference 20). Therefore, it would be useful to determine whether or not the high molecular weight protein associated with 10-nm filaments has properties similar to those of the MAPS of microtubules.

We are currently working to optimize and quantitate the disassembly-reassembly process. The system which we have described is well suited for such studies since disassembly occurs under physiological conditions, and harsh denaturation agents are not required. This should lead to a better understanding of the molecular architecture, function, and biochemical properties of 10-nm filaments.

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