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Isolation and preliminary characterization of 10-nm filaments from baby hamster kidney (BHK-21) cells

(colchicine/cell spreading/electron microscopy/polarized light/gel electrophoresis)

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ABSTRACT **A procedure for isolating 10-nm filaments as juxtanuclear caps from normal and colchicine-treated BHK-21 cells is described. These aggregates of 10-nm filaments retain their birefringence and their structural integrity. The major proteins comprising the filament preparations can be resolved as two bands on sodium dodecyl sulfate gels, corresponding to approximate molecular weights of 54,000 and 55,000.**

Microtubules (approximately 25-nm diameter), 10-nm filaments [about 7- to 12-nm diameter, also termed intermediate filaments(1)], and microfilaments (about 5- to 7-nm diameter) are the three types of cytoplasmic fibers that are found in animal cells (2–4). The structure, biochemistry, and *in vitro* assembly–disassembly of microtubules have been investigated intensively over the past decade and many functions appear to be directly related to these fibers (5, 6). A great deal of information is also available on the structure, function, and biochemistry of microfilaments, the major component of nonmuscle actomyosin-like contractile elements (3, 7–9). The 10-nm filament remains the least studied and the least understood of these fibers. The fibers are thought to be related to various physiological activities, including spreading and shape formation (10), intracellular organelle movements (11), and locomotion (4, 11, 12) in cultured cells. They are identical in morphology and size to the so-called tonofilaments (8–10 nm) frequently seen in association with desmosomes in epithelial cells (13, 14). In nerve axons, where they may be related to axoplasmic transport, they are called neurofilaments (15, 16). In smooth muscle cells, they are thought to be a component of the cytoskeletal system and are perhaps involved in dense body structure and function (17, 18).

Biochemical and structural characterizations of isolated 10-nm filaments have been attempted in only two systems: smooth muscle and the central nervous system. The results of studies on neurofilaments indicate that there are differences in both their sizes (7–10 nm) (1, 16, 19–23) and the molecular weights of their major protein subunits. Neurofilament subunits have reported molecular weights of 80,000 (20), 152,000, 160,000 (19), 50,000–53,000 (1, 16, 24), 57,000 (25), 212,000, 160,000, and 68,000 (15). These differences may be due to actual interspecies differences in protein composition or to proteolysis during fractionation (17). The biochemical data available on 10-nm filaments obtained from gizzard dense body preparations vary significantly from one laboratory to the next. The major polypeptides obtained in these preparations have molecular weights of 85,000, 105,000, 150,000 (17), 55,000 (18, 26), 50,000 (27), 115,000, 130,000, 160,000, and 200,000 (26). The discrepancies may reflect differences in fractionation and purification procedures.

One of the major problems encountered in isolating 10-nm filaments from mammalian systems may be due to the choice of experimental materials. Both smooth muscle and nervous tissue contain heterogeneous cell populations which impose the time-consuming steps of fractionation to obtain homogeneous cell types as in brain (1, 16, 21, 25), or to obtain complex organelles such as the dense bodies of gizzards (17, 18, 26, 28) prior to application of procedures to enrich 10-nm filaments. All techniques used on mammalian cells to date require anywhere from a few to 15 days to attain significant enrichment (1, 16–18, 21, 26, 28) of morphologically distinguishable filaments. The only exception to this is the highly specialized giant axon of the worm *Myxicola*, in which the axoplasm contains only neurofilaments and enriched preparations can be obtained in about 10 sec (19).

We have initiated a study which we feel eliminates many of the technical difficulties encountered in the isolation of 10-nm filaments from complex tissue systems. The study uses BHK-21 (baby hamster kidney) cells, in which large numbers of 10-nm filaments accumulate next to the nucleus in a birefringent sphere to form what has been termed a juxtanuclear cap during the early stages of cell spreading. As the spreading process progresses the cap of 10-nm filaments appears to "reel out" into birefringent streaks or fibers. The birefringent cap eventually disappears in fully spread cells and this is coincident with the redistribution of 10-nm filaments throughout the cytoplasm (3, 4, 10). Treatment of spread cells with colchicine (at concentrations sufficient to disassemble microtubules) leads to the formation of larger juxtanuclear caps of 10-nm filaments (3, 11, 14, 29, 30).

The preliminary results reported in this paper demonstrate that by isolating nuclei with attached filament caps, enriched preparations of filaments can be achieved following lysis of the nucleus. Remnants of nuclei remain as a significant contaminant when filament caps are prepared from normal, spreading cells, while much cleaner preparations are obtained from colchicine-treated cells. Alterations in the nuclear envelope [as evidenced by the extensive blebbing and pinching off of pieces of nuclear membrane (11)] may be responsible for making nuclei from colchicine-treated cells more susceptible to lysis. The caps of filaments retain their birefringent and ultrastructural properties. Furthermore, the caps contain two major bands of protein, as determined by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis, which can account for 80–90% of the total protein.

MATERIALS AND METHODS

Cell Cultures. Baby hamster kidney (BHK-21/C 13) cells were grown as described previously (11). Sub-confluent dishes

Abbreviations: BHK, baby hamster kidney; $NaDodSO_4$, sodium dodecyl sulfate; PBSa, phosphate-buffered saline (6 mM sodium–potassium phosphate buffer/171 mM NaCl/3 mM KCl, pH 7.1); DNase, deoxyribonuclease.

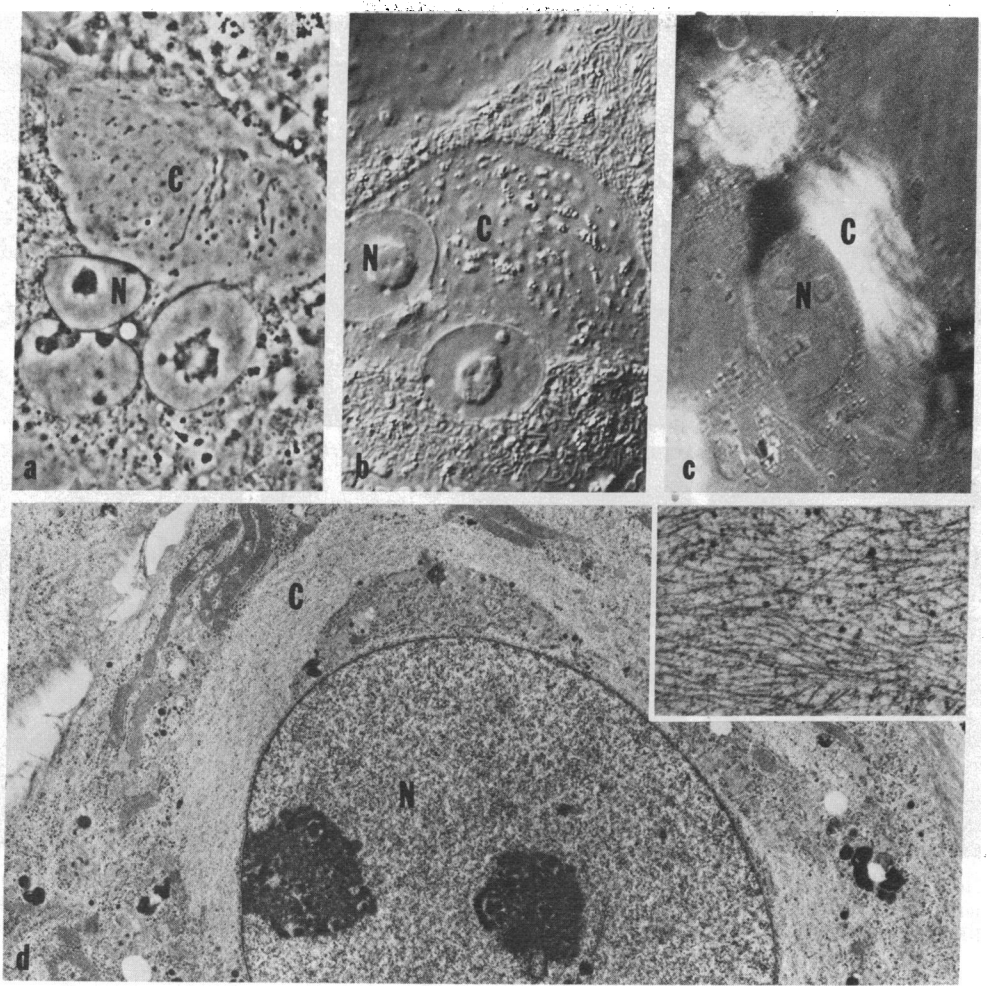

FIG. 1. Light and electron micrographs of BHK-21 10-nm filament caps *in situ* after 72 hr in colchicine (10 μg/ml). (*a–c*) Living cells grown on glass coverslips. N, nucleus; C, caps. (*a*) Phase contrast, ×920. (*b*) Nomarski, ×920. (*c*) Polarized light, ×650. (*d*) Electron micrograph of a thin section, ×5000. *Inset:* an enlargement of an area within the cap showing filaments approximately 9–10 nm in diameter, ×40,900.

of BHK-21 cells were treated with colchicine (Sigma) at 10 μg/ml in growth medium for 24–96 hr. Spreading populations of cells containing filament caps were obtained by trypsinizing and replating (11) confluent dishes of normal, untreated cells onto one-half of the number of starting dishes.

Isolation of Filament Caps. All steps were performed at 4° and centrifugations were at 2000 rpm for 3 min in a Dynac table-top centrifuge. All pellets were resuspended in a volume of 1 ml/100-mm dish of cells. Dishes of colchicine-treated (24–96 hr) BHK-21 cells were rinsed several times in phosphate-buffered saline without added Ca^{2+} or Mg^{2+} (PBSa; 6 mM sodium–potassium phosphate buffer/171 mM NaCl/3 mM KCl, pH 7.1), scraped off the dish with a rubber policeman, and centrifuged. The pellet was washed (resuspended in PBSa and centrifuged) and resuspended in PBSa. All the cells were broken by passage through a 26-gauge needle, as determined by phase contrast microscopy. The bulk of the cytoplasm was dispersed by this procedure and birefringent caps bound to nuclei were easily separated from other cytoplasmic particles by low speed centrifugation. The caps were morphologically identical to those seen *in vivo* (see Figs. 1 and 2) and thus polarized light microscopy was used throughout the procedure in an assay for the presence of 10-nm filament caps. The resulting suspension was centrifuged and washed, and the pellet was resuspended in deoxyribonuclease I (DNase, EC 3.1.4.5; Sigma) solution (0.05 mg/ml) containing 5 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonylfluoride (Sigma), a protease inhibitor, as a precautionary measure. There is no detectable difference in the $NaDodSO_4$ gel profile of purified filament caps when the protease inhibitor is omitted or when another (*p*-tosyl-L-arginine methyl ester·HCl) is added. The suspension was incubated at 37° for 30 min. and centrifuged. This pellet was washed in PBSa containing 5 mM EDTA (PBSa-EDTA), resuspended in 0.6 M KCl in PBSa-EDTA, and finally centrifuged and washed in PBSa-EDTA. At this point, most nuclei appeared to lyse completely. The prior treatment with DNase I was necessary to prevent the chromatin from forming large clumps with the filament caps. The caps were then resuspended in 1% (vol/vol) Triton X-100 in PBSa-EDTA, to remove membrane contamination, homogenized gently with three strokes in a ground-glass homogenizer, centrifuged, and washed four to five times in PBSa-EDTA. The resulting pellet was white, sticky, and difficult to resuspend. Very little nuclear contamination was seen in this final preparation as determined by phase contrast microscopy.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis was carried out on either 7.5% polyacrylamide slab gels with 4.5% stacking gels (31) with and without 6 M urea or on 5.6% tube gels (32). Protein concentration was determined by the microbiuret method (33). Chicken brain tubulin was purified with three cycles of assembly–disassembly (34). Microtubule polymerization was monitored by negative staining.

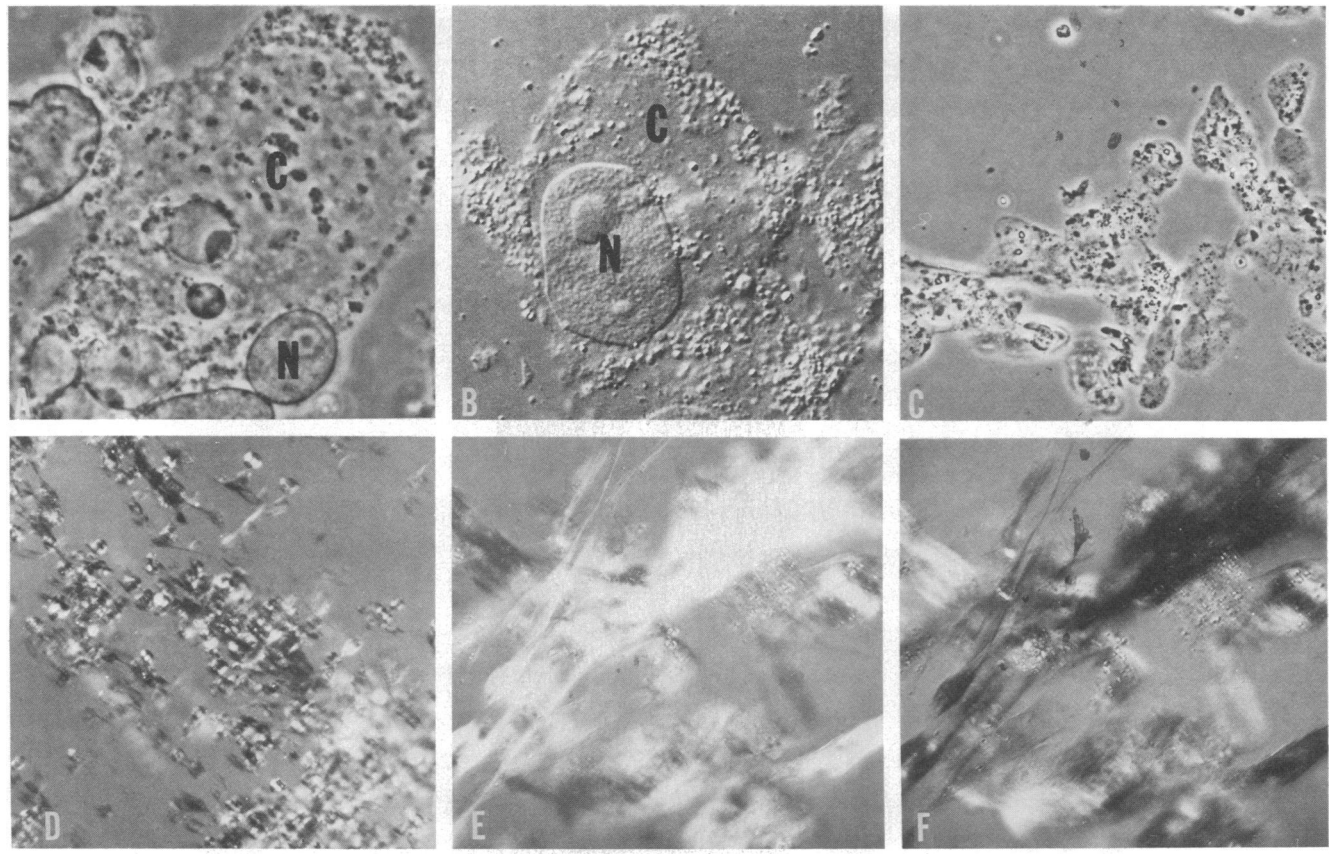

FIG. 2. Light micrographs of (*A* and *B*) lysed cells after syringing through a 26-gauge needle and (*C–F*) purified filament caps. Note how filament caps adhere to nuclei in *A* and *B*. N, nucleus; C, caps. (*A*) Phase contrast, ×1100. (*B*) Nomarski, ×1100. (*C*) Phase contrast, ×325. (*D*) Polarized light, ×100. (*E* and *F*) Same as *D* at opposite compensator settings, ×400. Note how the birefringence is retained in isolated caps (compare with Fig. 1*c*).

Light Microscopy. Living cells and stages in the isolation of filament caps were observed and photographed using a Zeiss Photomicroscope III equipped with phase contrast, polarized light, and Nomarski optics (11).

Electron Microscopy. Cells were fixed and flat embedded on plastic dishes (11). Isolated filament caps were fixed and embedded as pellets in capsules. Thin sections were made on an LKB Ultrotome III, mounted on uncoated grids, and stained with uranyl acetate followed by lead citrate (11). Negative stains were made on Formvar–carbon-coated grids with 3% aqueous uranyl acetate. Electron micrographs were taken on a Philips 201C electron microscope.

RESULTS

Colchicine-treated BHK-21 cells contain large juxtanuclear accumulations of 10-nm filaments (Fig. 1) that have been described in detail elsewhere (3, 4, 11). These filament caps, as they appear in the light microscope during the course of isolation, are shown in Fig. 2. Nuclei with attached filament caps are shown in Fig. 2 *A* and *B*. Fig. 2 *C–F* shows the purified filament caps devoid of visible nuclear contamination. Electron micrographs of thin sections and negative stains of purified filament caps appear in Fig. 3. The 10-nm filaments within the isolated caps are morphologically identical to those seen *in situ* (see Fig. 1*d*).

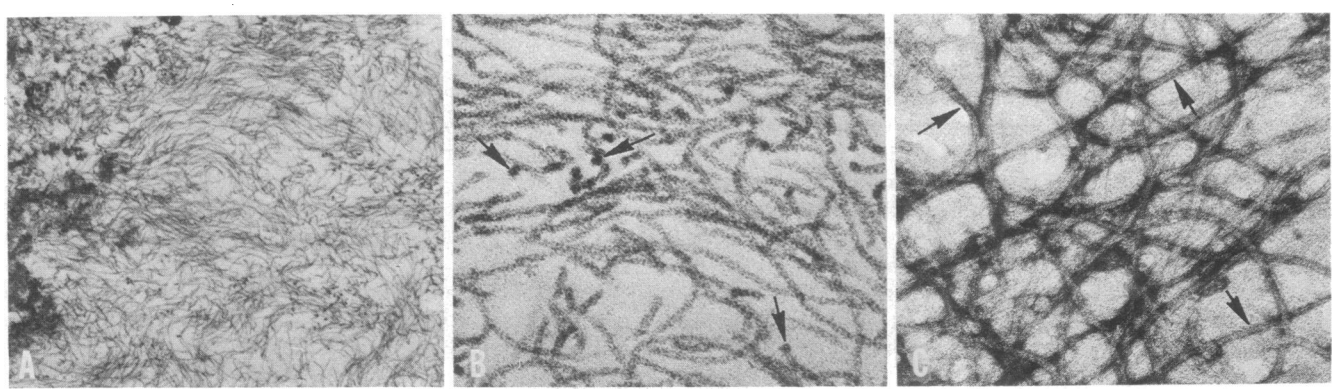

FIG. 3. Electron micrographs of purified 10-nm filament caps. Thin sections, (*A*) ×25,510, (*B*) ×121,600. Note cross sections of 10-nm filaments (arrows). (*C*) Negative stain of the edge of a cap showing filaments (arrows) ranging from 10 to 12 nm in diameter. The filaments are tangled and somewhat larger due to distortion and flattening as the sample dries on the grid. ×79,500.

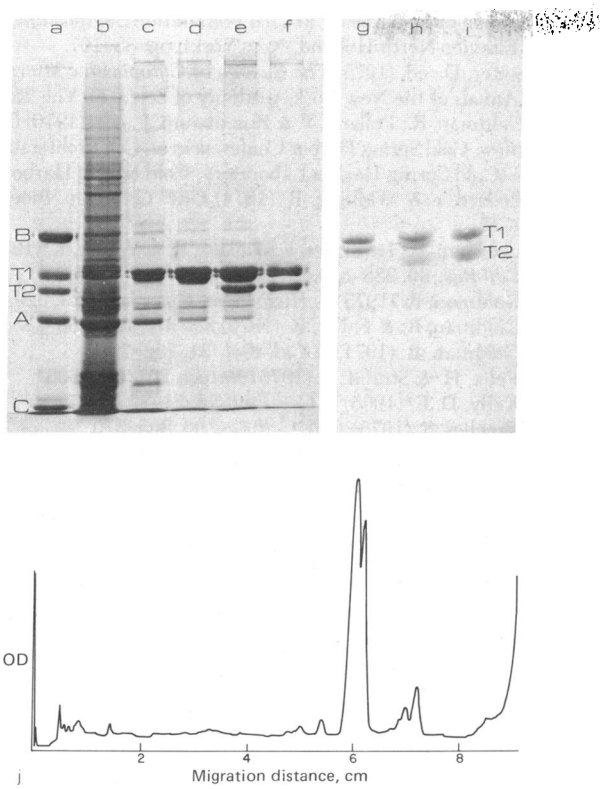

FIG. 4. NaDodSO$_4$/polyacrylamide slab gel (7.5%) with stacking gel (4.5%) (31) stained with 0.05% Coomassie blue R, 45% (vol/vol) methanol, 10% (vol/vol) acetic acid, and destained in 10% acetic acid. (*a*) Standards: bovine albumin (5 μg) (B), chicken brain tubulin (8 μg) (T_1-α, T_2-β), rabbit skeletal muscle actin (4 μg) (A), α-chymotrypsinogen (4 μg) (C). (*b*) BHK-21 whole cell protein (40 μg). (*c*) Filament cap protein from spreading cells (15 μg). (*d*) Filament cap protein from colchicine-treated cells (10 μg). (*e*) A mixture of filament cap protein from colchicine-treated cells (5 μg) and chicken brain tubulin (5 μg). α-Tubulin appears to comigrate with filament cap protein. (*f*) Chicken brain tubulin (10 μg). In *g–i*, 6 M urea was added to this gel system. (*g*) Filament cap protein from colchicine-treated cells (3.5 μg). (*h*) A mixture of filament cap protein from colchicine-treated cells and chicken brain tubulin (3.5 μg total). (*i*) Chicken brain tubulin (3.5 μg). α-Tubulin appears to migrate between the two bands of filament cap protein. (*j*) Scan of *d* on an E-C apparatus densitometer equipped with a 546 nm filter.

10-nm Filament caps from a normal, spreading population of cells [1.5–3 hr after plating (10)] were prepared by identical techniques. Very few nuclei disappear completely and the final preparations contain filament caps attached to nuclear remnants.

A NaDodSO$_4$/polyacrylamide slab gel prepared according to Laemmli (31) of isolated 10-nm filament caps from colchicine-treated and normal BHK-21 cells is shown in Fig. 4 (*c* and *d*). Approximately 80% of the total protein in the gel from the colchicine-induced caps is in two bands with approximate molecular weights of 54,000 and 55,000. These two bands comigrate with adult chicken brain α-tubulin, which also appears as two bands in this gel system. [The heterogeneity of α-tubulin has been found by others (35)]. The filament cap preparation from spreading cells shows enrichment (about 65% of protein in the gel) for the same two bands. There is no significant difference, other than relative amounts of minor components, between the two 10-nm filament cap protein preparations. The 55,000 and the 54,000 molecular weight components contain approximately 60% and 20%, respectively, of total cap protein. β-Tubulin appears to run with a molecular weight of 51,000. When 6 M urea is added to this gel system

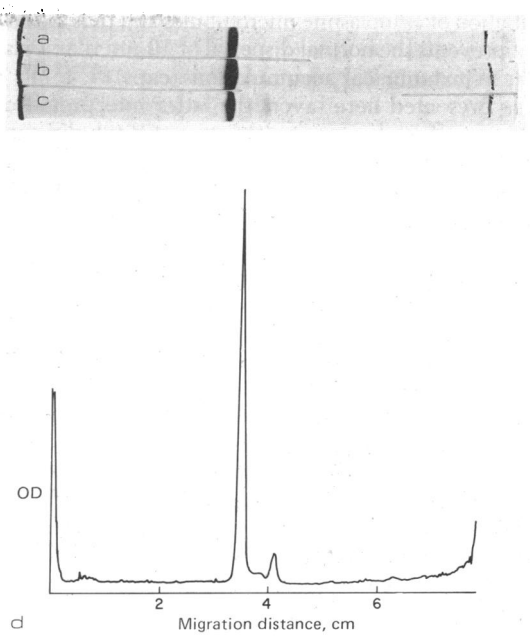

FIG. 5. NaDodSO$_4$/polyacrylamide tube gels (5.6%) (32) of (*a*) chicken brain tubulin (5 μg), (*b*) a mixture of filament cap protein (5 μg) and chicken brain tubulin (5 μg), (*c*) filament cap protein (5 μg). (*d*) A scan of *c* in a Gilford spectrophotometer at 550 nm.

(Fig. 4 *g–i*), α-tubulin is no longer resolved as a doublet and appears to migrate between the two bands of 10-nm filament cap protein. In addition, an antibody obtained against 10-nm filament cap preparations does not crossreact with tubulin, as assayed by double immunodiffusion and indirect immunofluorescence (J. M. Starger and R. D. Goldman, unpublished data). Similar immunological observations have been made by others (1, 16, 36).

NaDodSO$_4$ tube gels prepared according to Fairbanks *et al.* (32), of filament caps and chicken brain tubulin, are shown in Fig. 5. In this gel system the protein migrates as a single band with the same mobility as tubulin and constitutes about 90% of the total protein in the gel. It can be seen that the thin slab gel, utilizing the Laemmli technique (31), gives greater resolution. The average yield of protein from filament caps obtained following 96 hr of colchicine treatment is about 1.0 mg/40 mg of total cell protein.

The 10-nm filament cap preparations are soluble in 2 M urea, as determined by phase contrast and polarized light microscopy. Electron microscopic observations indicate that intact 10-nm filaments are no longer seen. The purified filament caps are resistant, however, to 0.6 M KCl treatment.

DISCUSSION

We have described a rapid (about 2 hr) technique for obtaining purified juxtanuclear 10-nm filament caps from colchicine-treated BHK-21 cells. To date, there has been no direct evidence, other than morphological, that the 10-nm filaments that accumulate in colchicine-treated cells are the same as those seen in normal cells. It is now apparent from this study that the major 10-nm filament protein subunits of normal BHK-21 cells have the same electrophoretic mobility as the subunits of 10-nm filaments accumulating in BHK-21 cells following colchicine addition. It has been suggested by several laboratories that these extensive arrays of 10-nm filaments seen after colchicine treatment may represent another polymerized form of tubulin (37). In BHK-21 cells, we have suggested that the intracellular distribution of 10-nm filaments is dependent upon a normal

distribution of cytoplasmic microtubules and that the colchicine effect prevents the normal dispersal of 10-nm filaments, which results in juxtanuclear accumulations (caps) (3, 4, 11). The evidence presented here favors the latter interpretation of the colchicine effect, because neither α- nor β-tubulin comigrates with 10-nm filament cap protein on $NaDodSO_4$/urea gels. Also, the two proteins appear to be immunologically distinct (refs. 1, 16, and 36; J. M. Starger and R. D. Goldman, unpublished data).

It is unclear at this point whether the minor bands seen on $NaDodSO_4$ gels are contaminants or integral components of filaments. It is possible that the small amount of protein comigrating with actin is a contaminant due to the presence of actin in large amounts in BHK-21 cells (about 8–10% of total cell protein, according to our unpublished observations).

The possible functions of 10-nm filaments in BHK-21 cell spreading and intracellular movements have also been discussed elsewhere (2–4, 10, 11). Microtubules and 10-nm filaments are in close proximity within the major cell processes of BHK-21 cells. Fine fibrillar material can be found, which appears to act as a bridge between the walls of microtubules and 10-nm filaments (2, 3). On the basis of these observations and others that demonstrate that mitochondria and other cytoplasmic particles exhibit saltatory movements along the long axes of major cell processes, we have suggested that a microtubule–10-nm filament complex might act as a two-component system involved in intracellular organelle movements (2, 11). The preparations of native filament caps afford us with the opportunity to study *in vitro* the microtubule–10-nm filament relationship by polymerizing microtubules in the presence of 10-nm filaments. These types of studies should provide new insight into the function and regulation of 10-nm filaments in mammalian cells.

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