A 300,000-mol-wt Intermediate Filament–associated Protein in Baby Hamster Kidney (BHK-21) Cells

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ABSTRACT Native intermediate filament (IF) preparations from the baby hamster kidney fibroblastic cell line (BHK-21) contain a number of minor polypeptides in addition to the IF structural subunit proteins desmin, a 54,000-mol-wt protein, and vimentin, a 55,000-mol-wt protein. A monoclonal antibody was produced that reacted exclusively with a high molecular weight (300,000) protein representative of these minor proteins. Immunological methods and comparative peptide mapping techniques demonstrated that the 300,000-mol-wt species was biochemically distinct from the 54,000- and 55,000-mol-wt proteins. Double-label immunofluorescence observations on spread BHK cells using this monoclonal antibody and a rabbit polyclonal antibody directed against the 54,000- and 55,000-mol-wt proteins showed that the 300,000-mol-wt species co-distributed with IF in a fibrous pattern. In cells treated with colchicine or those in the early stages of spreading, double-labeling with these antibodies revealed the co-existence of the respective antigens in the juxtanuclear cap of IF that is characteristic of cells in these physiological states. After colchicine removal, or in the late stages of cell spreading, the 300,000-mol-wt species and the IF subunits redistributed to their normal, highly coincident cytoplasmic patterns.

Ultrastructural localization by the immunogold technique using the monoclonal antibody supported the light microscopic findings in that the 300,000-mol-wt species was associated with IF in the several physiological and morphological cell states investigated. The gold particle pattern was less intimately associated with IF than that defined by anti-54/55 and was one of non-uniform distribution along IF, being clustered primarily at points of proximity between IF, where an amorphous, proteinaceous material was often the labeled element. Occasionally, "bridges" of label were seen extending outward from such clusters on IF. Gold particles were infrequently bound to microtubules, microfilaments, or other cellular organelles, and when so, IF were usually contiguous. During multiple cycles of in vitro disassembly/assembly of the IF from native preparations, the 300,000-mol-wt protein remained in the fraction containing the 54,000- and 55,000-mol-wt structural subunits, whether the latter were in the soluble state or pelleted as formed filaments. In keeping with the nomenclature developed for the microtubule-associated proteins (MAPs), the acronym IFAP-300K (intermediate filament associated protein) is proposed for this molecule.

Intermediate filaments (IF)[1] are a family of cytoplasmic fibrous elements that show similar ultrastructural morphologies, e.g., a 7–12-nm diameter, a coiled-coil, α-helical substructure, and a tubular cross-sectional profile (25, 26). In contrast to the evolutionary conservatism of the major structural subunits of microtubules and actinlike microfilaments, IF structural proteins can be divided into five general groups whose distribution in most cases is cell-type–specific on the basis of immunological and biochemical criteria (10, 25). This

[1] *Abbreviations used in this paper:* anti-54/55, rabbit antiserum against the 54,000- and 55,000-mol-wt BHK IF structural proteins; BHK cells, baby hamster kidney cells; IF, intermediate filaments; IFAP, IF-associated protein; IFAP-300K, a 300,000-mol-wt IFAP; MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino)ethane sulfonic acid; PBS_a, 6 mM Na^+-K^+ phosphate (pH 7.4), 171 mM NaCl, 3 mM KCl.

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diversity is especially apparent when one considers the number and types of structural subunits present in the various subclasses of IF. For example, a large number of keratin IF polypeptides have been identified in cells of various epithelial tissues, and the expression of specific keratins appears to be related to the type of epithelial cell and to the state of cellular differentiation (27, 42, 45). This complexity is compounded by the charge microheterogeneity displayed by most IF subunit proteins when analysed by two-dimensional gel electrophoresis (26, 43). Due to the apparent cell-type specificity, IF have been used as markers to determine cell type of origin in studies of differentiation and tissue pathology (e.g., neoplasia) (30). While potentially very useful in clinical pathology and studies of differentiation, for example, there is some ambiguity introduced because of the apparent co-existence of more than one IF class in a single cell type (9, 12, 13) and the presence in some cell types of an IF class that is seemingly inconsistent with their embryological origins (11, 34, 46, 47, 55).

Little is known about the regulatory mechanisms involved in IF assembly/disassembly or IF organization within cells. IF have phosphorylated subunits (3, 29), and the phosphorylation of two IF proteins, desmin and vimentin, by cAMP-dependent protein kinases has been demonstrated in vitro (14). Moreover, several calcium-activated proteases that specifically degrade IF proteins have been reported (28, 31). While such factors have been shown to be instrumental in the functional regulation of many other proteins, direct evidence for their involvement in IF function is lacking. In this regard, attention has only recently begun to be directed to the numerous minor polypeptide species detectable in IF preparations from most cells types.

Several such proteins have been investigated from the standpoint of their being IF-associated. One of the most extensively studied examples is a minor, high molecular weight (230,000) protein named synemin that was originally identified by its association with IF in chicken smooth muscle and erythrocytes (17, 19). It has been proposed that this protein cross-links IF. In conjunction with another IF-associated protein termed paranemin (2), synemin exhibits a complex developmental expression related to the desmin and/or vimentin composition of the IF of chicken muscle and some nonmuscle cells (32). A group of high molecular weight polypeptides associated with the respective IF of several cultured cell lines has similarly been identified (33). An unusual, low molecular weight (~30,000) class of proteins associated with keratin-containing IF is filaggrin (5, 39). Other recently identified low molecular weight examples include p50, a 50,000-mol-wt protein identified by a monoclonal antibody to be associated with vimentin-containing IF in a variety of cell types (48), and epinemin, a 45,000-mol-wt vimentin-associated protein of wide cell-type distribution (24). Some of these proteins may play important roles in determining the nature of IF organization in cells, as well as in regulating the numerous interrelations that have been observed by microscopic methods between IF and other cellular elements and among IF themselves (16, 21). It is becoming increasingly obvious that these close spatial arrangements reflect functional interactions.

IF prepared from BHK-21 cells have been extensively characterized with respect to their polypeptide staining pattern on polyacrylamide gels (38). The major species possess molecular weights of 54,000 (desmin) and 55,000 (vimentin), and represent the structural subunits of the cells' IF. On the basis of molecular weight, there are two main groups of minor polypeptides, a 60,000–70,000-mol-wt group (15, 55) and a 200,000–300,000-mol-wt group (38, 53). In the present study, a monoclonal antibody has been produced that is specific for a 300,000-mol-wt component of native IF from BHK-21 cells. Immunofluorescence and ultrastructural immunogold localizations using this antibody suggest that the 300,000-mol-wt protein is an intermediate filament–associated protein (IFAP). This morphological evidence is supported by in vitro disassembly/assembly studies showing that, unlike some other minor species, the 300,000-mol-wt protein segregates with the IF during this procedure. The acronym IFAP-300K is proposed for the protein.

MATERIALS AND METHODS

Cell Culture: Baby hamster kidney (BHK-21/C13) cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum, 10% tryptose phosphate broth, 50 U/ml penicillin and 50 μg/ml streptomycin. Stock cultures in 100-mm plastic tissue-culture dishes were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Confluent cultures were transferred to other dishes or roller bottles after treatment with 0.05% trypsin-EDTA solution (Gibco Laboratories).

Cells fixed at various stages during spreading were used for microscopic studies. Some subconfluent dishes of cells were treated with medium containing 10 μg colchicine/ml for 12–24 h. For reversal studies, cells were incubated in normal culture medium for another 24 h after colchicine treatment.

Isolation of BHK Cell IF: BHK IF were isolated by the procedure of Zackroff and Goldman (54) as modified from Starger et al. (38). Confluent roller bottles were rinsed three times with phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS_a: 6 mM Na^+-K^+ phosphate [pH 7.4], 17 mM NaCl, 3 mM KCl). 10 ml of cold lysis buffer (0.6 M KCl, 10 mM $MgCl_2$, 1% Triton X-100 in PBS_a) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM p-tosyl-L-arginine methyl ester were added to each bottle. The lysed cells were detached by rolling the bottles at room temperature for 5 min. The cell suspension was homogenized with three strokes in a glass homogenizer. DNase I (Sigma Chemical Co., St. Louis, MO) was added to a concentration of 0.5 mg/ml and the suspension was incubated for 5 min at 4°C. The solution was then centrifuged at 2,200 *g* for 5 min at 4°C. The resulting pellet of native IF was washed three times in PBS_a containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride to remove excess salts and the remaining detergent. IF pellets at this stage were used for monoclonal antibody production or for disassembly/assembly studies, while those for SDS PAGE were given a final wash in 10 mM Tris-HCl (pH 7.4). Protein concentrations were determined by the method of Bradford (1), or by absorbance at 280 nm. Lyophilized native BHK IF was used as a standard.

Disassembly/Assembly Cycling of IF: Cycling of native preparations of BHK IF was performed essentially by the procedure of Zackroff and Goldman (54) following the observation by Starger et al. (38) of the reversible assembly properties of this class of IF. This consisted of dialysis at 4°C of an homogenate of the IF preparation against low salt buffer (5 mM Na^+ phosphate [pH 7.2], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) for 18 h (100 vol with two changes). The material was clarified by centrifugation at 265,000 *g* for 1 h at 4°C in a Beckman type SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). The resultant supernatant was designated S_1. IF assembly was induced by the addition of 0.09 vol of 1.9 M NaCl in low salt buffer. The assembly process was allowed to proceed at room temperature for 2 h, at which time the polymerized IF were pelleted by centrifugation at 157,000 *g* for 45 min at 23°C. The pellet (P_1) represented once-cycled IF. The procedure was repeated to yield twice-cycled IF (P_2).

PAGE: SDS PAGE was performed on slab gels by the method of Laemmli (23) using either 5% or 7.5% acrylamide separating gels and a 4.5% stacking gel. IF specimens for electrophoresis were dispersed in 10 mM Tris-HCl (pH 7.4), at a concentration of ~1 mg/ml. Concentrated SDS-β-mercaptoethanol (Sigma Chemical Co.) buffer was then added to yield a final protein concentration of 0.7 mg/ml. Gels were stained with 0.1% Coomassie Blue R in 50% methanol and 10% acetic acid and destained in 10% acetic acid. Molecular weights were determined by calibration with commercial standard kits (Bio-Rad Laboratories, Richmond, CA) on both 5% and 7.5% gels.

Monoclonal Antibody Production: Six BALB/c female mice were immunized with the native IF preparations described above. The immu-

nization schedule comprised an initial injection of 0.4 mg of total IF pellet emulsified in an equal volume of Freund's complete adjuvant (Gibco Laboratories) and two subsequent boosts of 0.2 mg of IF preparation emulsified in an equal volume of Freund's incomplete adjuvant (Gibco Laboratories). Blood samples were collected and tested by immunofluorescence on BHK-21 cells and by immunoblotting using the procedure of Towbin et al. (44). 2 d after the second boost, the mice that had given the strongest immunoreaction with the proteins of interest were killed and their spleens removed. Spleen cells were fused with the mouse myeloma cell line Sp2/0 according to established procedures (22, 36). The cells were aliquoted into twenty 24-well culture dishes (Costar, Cambridge, MA). Culture supernatants from surviving clones were first assessed for antibody content by immunofluorescence and the positive ones were further tested by immunoblotting. Three samples of hybridoma cells from all the immunofluorescence-positive wells were stored frozen in liquid nitrogen. The hydridomas were then cloned three times in succession in 96-well plates by the technique of limiting cell dilution. Culture supernatants from the resulting subclones were tested by both immunofluorescence and immunoblotting.

Identification of Monoclonal Antibody Subclass: The antibodies present in the culture supernatants of the hybridoma cell lines were identified according to immunoglobulin subclass by the enzyme-linked immunosorbent assay using commercial mouse immunoglobulin subtype identification kits (Boehringer Mannheim Biochemicals, Indianapolis, IN). Low salt–solubilized (5 mM Tris-HCl [pH 7.4]) IF preparations were coated on microplates and incubated successively with culture supernatants, rabbit anti-mouse antibody, and peroxidase-conjugated goat anti-rabbit IgG. The reactions with 2,2-azino-di-[3-ethyl-benzthiazoline sulfonate]/H_2O_2 solution were quantitated by measuring the optical density of each well at 415 nm.

Immunoblotting: After SDS PAGE of solubilized IF, one sample lane was sliced from the gel and stained for reference purposes. The polypeptides in the unstained portion were electrotransferred onto a sheet of nitrocellulose paper by the method of Towbin et al. (44). The effectiveness of a transfer was assessed by staining a vertical strip of the paper (one sample lane) for 5 min in 0.1% amido black in 5% methanol and 10% acetic acid, followed by a 15-min de-stain in 5% methanol and 10% acetic acid. The remainder of the nitrocellulose sheet was then incubated in a solution of 10% fetal calf serum, 2.5% bovine serum albumin in PBS_a for 1 h, rinsed briefly in PBS_a, and then cut into strips that were incubated overnight with the monoclonal antibodies or a rabbit antiserum against the 54,000- and 55,000-mol-wt BHK IF structural proteins (anti-54/55). The strips were washed with five changes of PBS_a over 15 min, and incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Miles-Yeda, Ltd., Israel) diluted 1:1,000 in PBS_a. After being washed with five changes of PBS_a, the strips were developed in freshly prepared 0.05% 4-chloro-1-naphthol (diluted from fresh 0.3% stock in methanol) and 0.01% H_2O_2 in PBS_a. The enzyme reaction was stopped by H_2O rinses, and the nitrocellulose strips were air-dried and photographed.

Peptide Mapping: One-dimensional peptide mapping by limited proteolysis was performed by the technique of Cleveland et al. (4). SDS-solubilized native BHK IF were electrophoresed on the entire widths of SDS polyacrylamide gel slabs cast without formed lanes. After the staining of each gel slab for 20 min with Coomassie Blue, protein bands of interest were sliced in full-width horizontal strips and stored at −80°C in 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and 1 mM EDTA overnight. Proteolytic digestion of proteins from the gel slices was achieved with the protease *Staphylococcus aureus* V8 (Miles Laboratories Inc., Elkhart, IN).

Amino Acid Analysis: Bands of protein were sliced from SDS polyacrylamide gels as described for peptide mapping (see above). The slices were coarsely minced in ~3 ml of 1 mM NH_4HCO_3 per slice (representing one complete gel slab) and dialyzed vs. several changes of the same solution. The minces were then centrifuged at 10,000 *g* and the eluted protein precipitated from the supernatant with 6 vol of cold acetone. The protein was air-dried and dispersed in 0.3 ml of 6 N HCl. Hydrolysis was performed in sealed tubes at 108°C for 24 h. The dried hydrolysate was analyzed in a JEOL Model 6AH amino acid analyzer (JEOL USA, Cranford, NJ).

Indirect Immunofluorescence: Cells grown to the desired density on glass coverslips were rinsed in PBS_a, fixed for 3 min in ice cold methanol, and air-dried. Each coverslip was overlaid with 80 μl of monoclonal culture supernatant and incubated in a humidified chamber for 1 h at 37°C. After incubation, they were washed with PBS_a and then incubated for 1 h at 37°C with fluorescein- or rhodamine-conjugated goat anti-mouse IgG/IgM (1:30 dilution) (Miles-Yeda, Ltd., Israel). Coverslips were washed extensively with distilled water and mounted on glass slides in Gelvatol (Monsanto Co., St. Louis, MO). Culture supernatants of nonproductive hybridoma cell lines and rabbit anti-54/55 were used as controls. For double-label immunofluorescence, a mixture of anti–IFAP-300K (1:5 final dilution) and rabbit anti-54/55 (1:60 final dilution) was applied as the primary antibody solution, and a mixture of rhodamine-conjugated goat anti-mouse IgG (1:10 final dilution) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and fluorescein-conjugated goat anti-rabbit IgG (1:30 final dilution) (Miles-Yeda, Ltd.) was applied as the secondary antibody solution.

Controls for cross-reactivity between the two secondary antibodies used for double immunofluorescence (anti-mouse IgG and anti-rabbit IgG) consisted of incubating coverslips with either anti–IFAP-300K or anti-54/55, followed by incubation with a mixture of both secondary antibodies. Other controls included the preabsorption of antibody preparations with antigen. These were performed on anti–IFAP-300K by incubating monoclonal culture supernatants with nitrocellulose strips containing electrotransferred IFAP-300K. The residual supernatant was then used to stain cells as described above.

Cells were examined with a Zeiss Photomicroscope III equipped with a IIIRS epifluorescence system (Carl Zeiss, Inc., Thornwood, NY). Fluorescence micrographs were taken with Kodak Plus-X film (Eastman-Kodak Co., Rochester, NY) utilizing a xenon lamp for fluorescein visualization and a mercury lamp for rhodamine. Photomicrographs were developed in Diafine (Acufine, Inc., Chicago, IL).

Electron Microscopy: Pellets of BHK IF preparations were fixed in 1% glutaraldehyde in PBS_a for 30 min, washed in PBS_a, and postfixed in 1% OsO_4 in PBS_a for 30 min. After en bloc staining with 3% uranyl acetate for 30 min, the pellets were dehydrated and embedded in Epon-araldite. Thin sections were mounted on uncoated grids and stained with 3% uranyl acetate for 20 min, followed by lead citrate for 10 min. Electron micrographs were taken on a JEOL 100CX electron microscope at an accelerating potential of 60 kV (JEOL USA, Peabody, MA).

Ultrastructural Immunogold Localization: For the ultrastructural localization of IFAP-300K by the immunogold technique (7), we processed BHK-21 cells grown in 35-mm culture dishes by the following protocol using a 2-(N-morpholino) ethane sulfonic (MES) acid (Research Organics Inc., Cleveland, OH) buffer system: (*a*) Three rapid washes in PBS_a. (*b*) Fixation for 15–30 s in 0.07–0.1% glutaraldehyde, 0.15% Triton X-100 in MES buffer (0.1 M MES [pH 6.6], 0.5 mM $MgSO_4$, 2 mM EGTA [for microtubule preservation, 30% glycerol was included]). (*c*) Three washes in MES buffer for 10 min (total time). (*d*) Treatment with 0.15% Triton X-100 in MES buffer for 2 min to further permeabilize the fixed cells. (*e*) Three washes in MES buffer for 10 min (total time). (*f*) Treatment with 0.5 mg/ml $NaBH_4$ in MES buffer for 20 min to reduce free aldehyde groups on glutaraldehyde that can nonspecifically bind colloidal gold. (*g*) Five washes in MES buffer for 20 min (total time). (*h*) Incubation for 10 min at 37°C with normal goat serum diluted 1:30 in MES buffer containing 0.1% bovine serum albumin to block nonspecific binding sites on proteins. (No wash followed this step, the serum was simply pipetted off.) (*i*) Incubation with primary antibody (anti–IFAP-300K or anti-54/55) for 50 min at 37°C in a moisture chamber. (*j*) Five washes with MES buffer on a rotary shaker for 30 min (total time). (*k*) Incubation with normal goat serum (as in *h*). (*l*) Incubation with 5 nm colloidal gold–conjugated goat anti-mouse or anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium) for 50 min at 37°C in a moisture chamber. (*m*) A 30-min wash in MES buffer on a rotary shaker.

The specimens were then fixed in glutaraldehyde-osmium tetroxide, embedded and stained for electron microscopy as described above. With the substitution of the appropriate fluorochrome-conjugated secondary antibody for the colloidal gold conjugate in *l*, we also used this protocol to prepare samples for immunofluorescence (20). (For this purpose, *f* may be omitted without altering the results.)

RESULTS

Polypeptide Composition of Native IF Preparations

In agreement with earlier studies (38, 54), the polypeptide composition of IF preparations from BHK-21 cells was represented in SDS PAGE primarily (~80%) by the structural IF subunits desmin, a 54,000-mol-wt protein, and vimentin, a 55,000-mol-wt protein (Fig. 1*A*). A group of related polypeptides (with molecular weights of 60,000–70,000) that have been shown to be keratinlike (15, 55) represented ~10% of the total protein content of the preparation. A prominent 300,000-mol-wt doublet species comprised ~3%, while multiple 40,000–50,000-mol-wt polypeptide bands and several minor high molecular weight polypeptides comprised the balance of the protein. The 40,000–50,000-mol-wt region has been shown to contain, among other elements, proteolytic

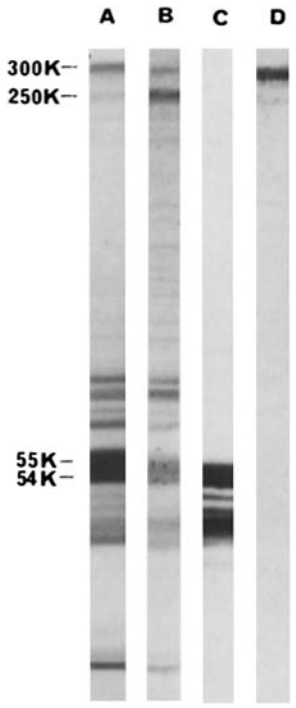


FIGURE 1 Native BHK IF composition and monoclonal anti-IFAP-300K characterization. (*A*) The polypeptide composition of a representative native BHK cell IF preparation shown on a Coomassie Blue-stained, 7.5% polyacrylamide SDS gel. Molecular weights × 10^{-3} of significant polypeptide bands are indicated. (*B*) Amido black-stained nitrocellulose transfer of *A*. (*C*) Nitrocellulose transfer of *A* immuno-stained with rabbit antiserum directed against 54/55. (*D*) Nitrocellulose transfer of *A* immuno-stained with a monoclonal antibody directed against IFAP-300K. No cross-reaction between the two sets of antigens was detectable.

derivatives of the 54,000- and 55,000-mol-wt subunits (37). It was the minor species co-isolating with the two major IF subunits that were of interest as potential IF-associated proteins.

Characterization of a Monoclonal Antibody Directed against IFAP-300K

In this study, six female BALB/c mice were immunized with native BHK IF preparations. Two mice whose sera were immunologically responsive to the minor species in this antigen mixture were selected. After fusion of their excised spleen cells with mouse myeloma cells to form hybridomas, numerous monoclonal antibodies directed against different polypeptides of the native BHK IF preparations were produced. Preliminary screening determined that one of these antibodies, shown by the enzyme-linked immunosorbent assay technique to be of the IgG_{2b} immunoglobulin subclass, gave an IF staining pattern by immunofluorescence microscopy and reacted with a 300,000-mol-wt polypeptide band on immunoblots. This monoclonal antibody was selected as a probe for further investigation of an apparent IF-associated protein (IFAP-300K) and was designated anti-IFAP-300K.

SDS PAGE of native IF showed that IFAP-300K was represented by a doublet band (Fig. 1*A*). The monoclonal antibody reacted exclusively with this doublet on immunoblots of these gels (Fig. 1*D*). Rabbit antiserum directed against the 54,000- and 55,000-mol-wt structural subunit proteins did not cross-react with IFAP-300K (Fig. 1*C*). Anti-54/55 also reacted with protein bands in the 40,000–50,000-mol-wt range. These most probably represented the proteolytic breakdown products of 54/55 described earlier (38). Immunoblots of heavily loaded SDS gels occassionally revealed a faint reaction between anti-IFAP-300K and a 250,000-mol-wt band (Fig. 1*D*).

Biochemical Analyses of IFAP-300K

The relationship among these polypeptides was further investigated by biochemical methods. Five bands (each of the IFAP-300K doublet bands, and the 250,000-, 55,000-, and 54,000-mol-wt bands) were cut from several SDS gels of native IF preparations for one-dimensional peptide mapping by limited proteolysis (4). The patterns generated from each of the 300,000-mol-wt species and the 250,000-mol-wt band were very similar (Fig. 2, *A–C*) but showed practically no homology with the maps of either the 55,000- or 54,000-mol-wt species. (Fig. 2, *D* and *E*). Amino acid analysis of IFAP-300K showed that its composition was similar to that of the 54,000- and 55,000-mol-wt structural subunits (Table I) and suggested that it was, like the structural IF proteins, an acidic species (Glu and Asp ~27 mol%; His, Lys, and Arg, ~13 mol%). Collectively, these data demonstrated that IFAP-300K was distinct from the 54,000- and 55,000-mol-wt subunits.

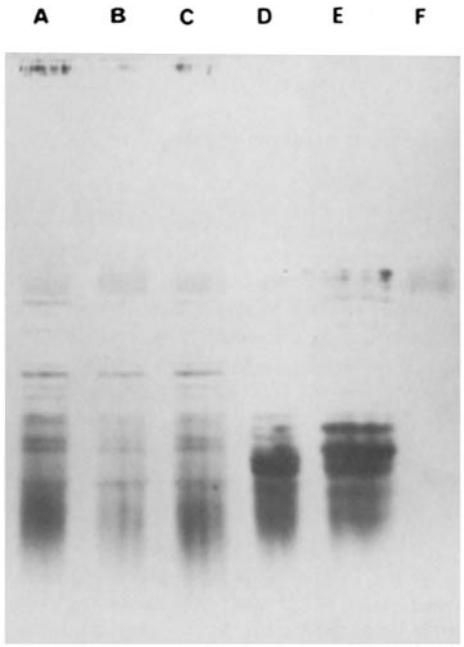


FIGURE 2 Comparative one-dimensional peptide mapping of native IF polypeptides. Polypeptide bands sliced from SDS gels of native BHK IF preparations were digested in situ on 15% polyacrylamide SDS gels. Coomassie Blue-stained. (*A*) Upper band of IFAP-300K doublet. (*B*) Lower band of IFAP-300K band. (*C*) 250,000-mol-wt polypeptide. (*D*) 55,000-mol-wt polypeptide. (*E*) 54,000-mol-wt polypeptide. (*F*) V8 protease. The IFAP-300K and 250,000-mol-wt polypeptides gave identical patterns that showed no homology with either the 54,000- or 55,000-mol-wt polypeptide patterns. Various quantities of V8 protease (1 ×, 3 ×, and 5 × the ratio recommended by Cleveland et al. [4]) were tested on each band, but aside from more complete digestion of the respective, original protein, no qualitative differences were detectable in the mapping patterns obtained for each protein with these different enzyme/substrate ratios.

TABLE I

Amino Acid Composition of BHK Cell IFAP-300K and IF Polypeptides

	Mol %		
	IFAP-300K	55,000-mol-wt polypeptide	54,000-mol-wt polypeptide
Asp	9.69	11.58	9.75
Thr	5.76	5.30	4.63
Ser	6.69	6.96	9.90
Glu	17.59	17.88	16.13
Pro	6.82	3.93	5.78
Gly	8.90	6.85	7.72
Ala	9.97	9.08	9.02
Val	3.23	5.23	6.02
Met	2.17	1.22	0.90
Ile	1.96	3.10	3.00
Leu	9.21	10.45	7.75
Tyr	2.49	2.03	2.24
Phe	2.50	2.89	2.92
His	2.23	1.65	0.66
Lys	3.93	4.36	4.45
Arg	6.86	7.48	8.95

Values are averages of two 24-h hydrolyses for each protein. Cys and Trp values were not determined.

By the same criteria, the 250,000-mol-wt protein was closely related to IFAP-300K and probably represented a proteolytic breakdown product of IFAP-300K. This conclusion was supported by variability in the amount of the 250,000-mol-wt protein detected in native IF prepared on different occasions.

Immunofluorescence Evidence for 300K/IF Association

The subcellular localizations of IF and IFAP-300K in BHK-21 cells were determined by single and double immunofluorescence labeling using rabbit anti-54/55 and monoclonal anti-IFAP-300K as probes. In general, these antibodies demonstrated the existence of a very close morphological association between the respective antigens in all physiological and experimental states studied. In cells fixed during the early stages of spreading (i.e., posttrypsinization and replating), the majority of anti-54/55 fluorescence was concentrated in a juxtanuclear cap within each cell (Fig. 3*A*). A similar cap was revealed with double-label staining by anti-IFAP-300K (Fig. 3*B*). During later stages of cell spreading, this overall correspondence of the two patterns was maintained; both antibodies revealed the typical IF network of spread cells (Fig. 3*C–F*). It should be noted that in some preparations (e.g., in Fig. 3, *C* and *D*) there was a slightly punctate nature to the anti-IFAP-300K filamentous pattern compared with the continuous filamentous pattern observed for anti-54/55 staining, perhaps suggesting an intermittent or periodic distribution of IFAP-300K in IF networks.

Double-labeling of colchicine-treated cells further emphasized the interrelationship between IF and IFAP-300K. Thus, a majority of both antigens retracted into the juxtanuclear arrays characteristic of colchicine-treated cells (Fig. 3, *G* and *H*). After removal of the drug, the two staining patterns once again appeared coincidently dispersed throughout the cytoplasm (Fig. 3, *I* and *J*). These data strongly suggested an association between IF and the 300,000-mol-wt protein. Especially convincing of this point was the dynamic nature of the coincidence of the two staining patterns; i.e., the persistence of co-distribution during the transition of IF from dispersed networks to juxtanuclear caps, back to dispersed networks under both physiological and drug-induced experimental conditions. All controls for these immunofluorescence observations (see Materials and Methods) were negative.

300K/IF Association As Determined by Immunogold Labeling

We used the indirect immunogold method to determine more precisely the nature of the association between IFAP-300K and IF as suggested by the immunofluorescence results. By simultaneously permeabilizing and fixing the cells for brief periods (see Materials and Methods), we preserved many intracellular organelles, thus making it possible to localize IFAP-300K in relation to these other structures in situ and to compare its distribution with that of the structural IF subunits.

The results showed that both the anti-IFAP-300K and the anti-54/55 were morphologically associated with IF (Figs. 4*A* and 5–7), thus corroborating the co-distribution of the two antigens as detected by immunofluorescence (Fig. 3). More specifically, comparison of Fig. 4*A* with *B* demonstrates the distinctly different antigen localization along the IF obtained with anti-IFAP-300K and anti-54/55 gold labeling, respectively. The antibody against 54/55 was found immediately adjacent to IF and appeared to be associated with them along their entire lengths. In contrast, the antibodies that reacted with IFAP-300K were not located in a continuous pattern along the IF; instead they were present in randomly spaced clusters. These foci of gold particles were often found at points of proximity between IF (Fig. 4*A* and 5). In some cases, the label at these points was localized to amorphous, proteinaceous material that, in turn, was closely associated with IF (Figs. 4*A*, 6 and 7). The association between the anti-IFAP-300K label and IF was not as intimate as that seen between the 54/55 label and IF. In many instances, the 300,000-mol-wt label appeared to extend from a cluster clearly located on one IF toward another IF, or sometimes to no apparent structure at all. The latter observation could have been due to the fact that other IF/IFAP-300K complexes that possessed gold labels were out of the plane of the thin section and therefore could not be visualized.

The patterns of localization of IFAP-300K/IF just described were primarily studied in cells containing dispersed networks of IF (Figs. 4 *A*, 5). The association also existed in several other organizational states assumed by BHK IF under normal and experimental conditions. Thus, in juxtanuclear caps formed after trypsinization/replating or colchicine treatment of the cells, gold particles were similarly found in focal locations on the IF (Fig. 6). Observations on other IF organizational states served to highlight both the persistence of the IFAP-300K/IF association, as well as the different nature of the IFAP-300K association to IF compared with that of the 54,000- and 55,000-mol-wt subunits. For example, Fig. 7 demonstrates a loose bundle of IF fibers to which the anti-IFAP-300K label was associated in what appeared to be a position around the whole of the bundle rather than with any component filament.

Although the gold labeling data indicated a less intimate relationship between IFAP-300K and IF than the obvious one between the structural 54,000- and 55,000-mol-wt subunits and IF, in all instances the IFAP-300K probe more closely

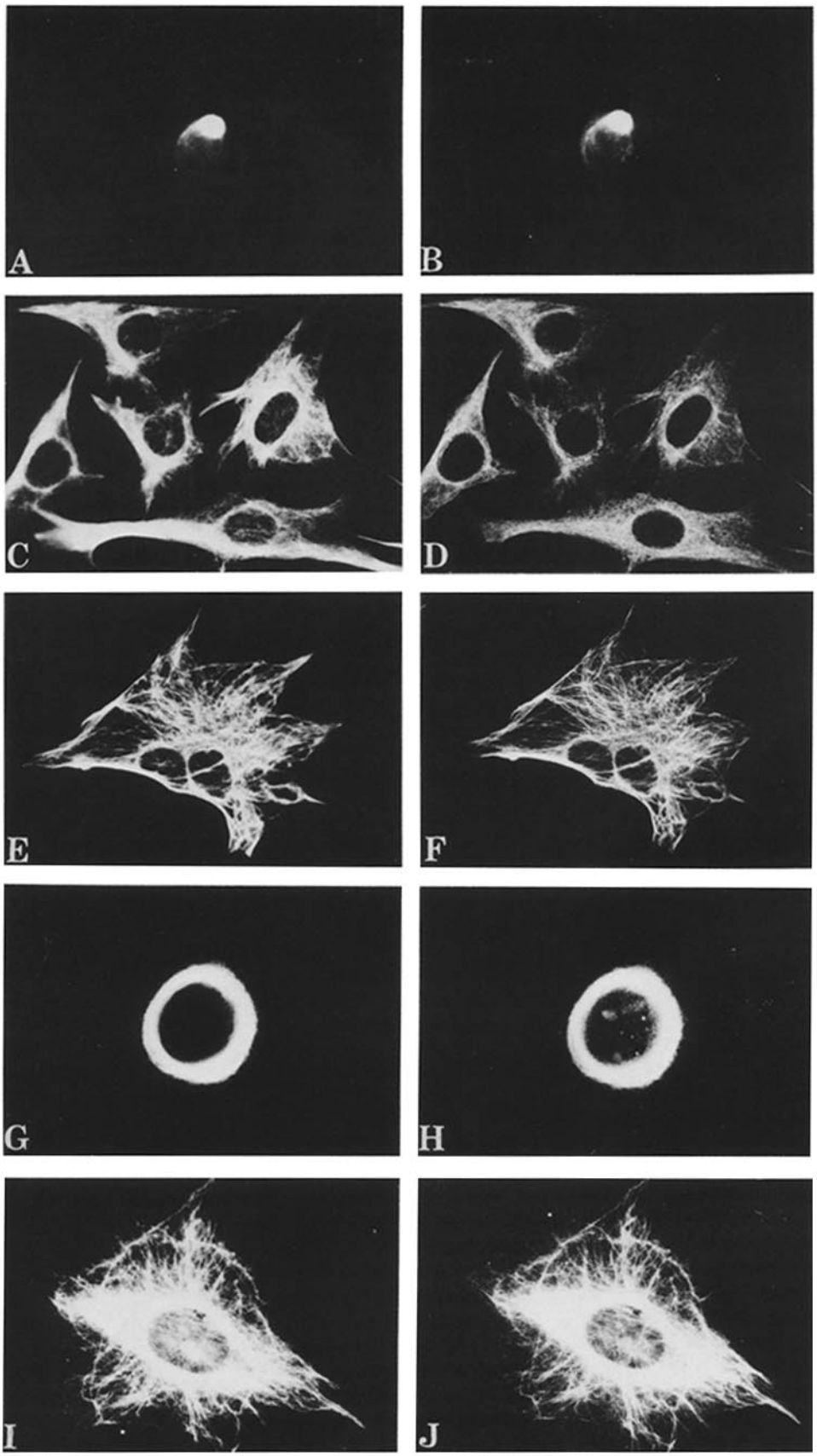
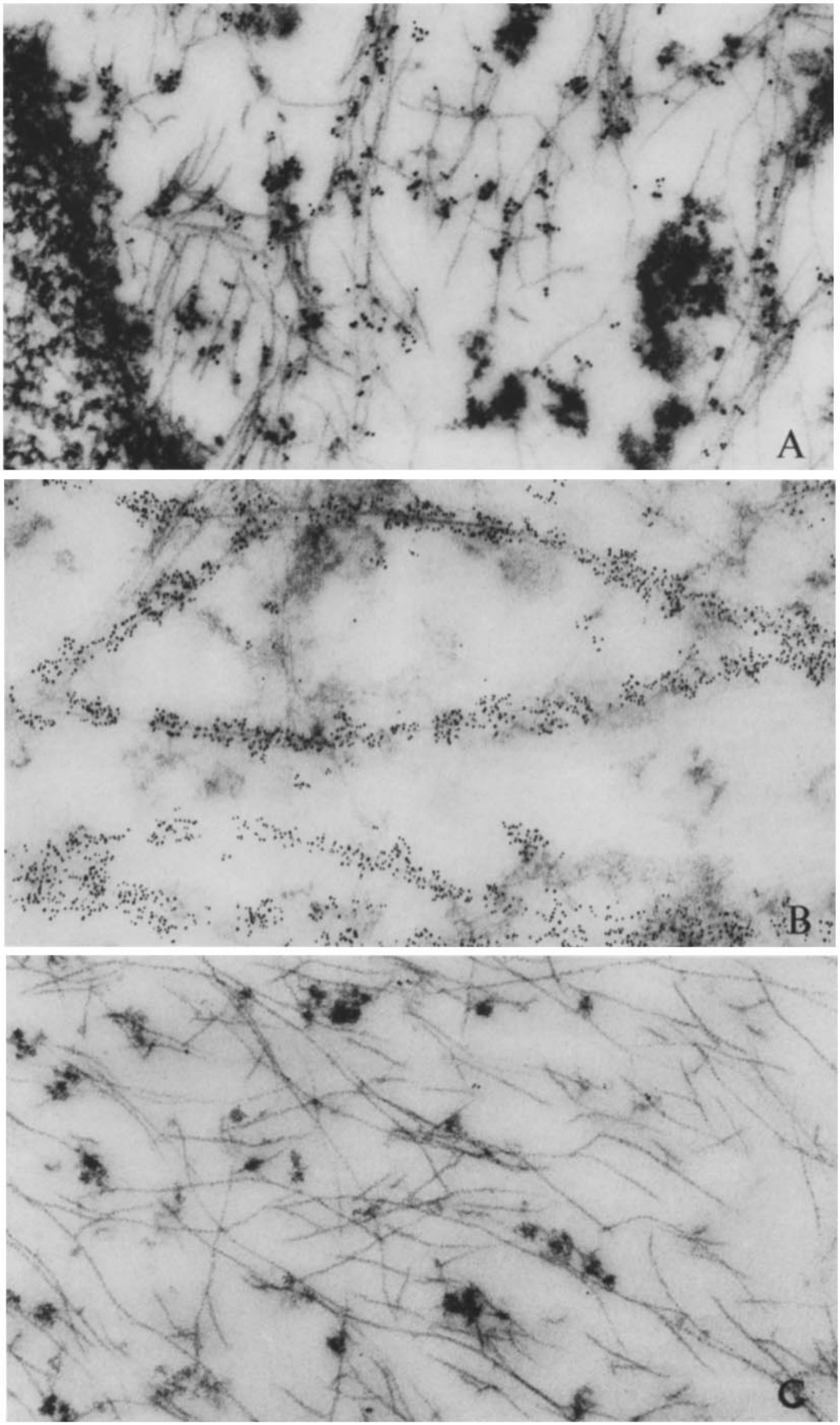


FIGURE 3 Double label immunofluorescence localization of IFAP-300K, and 54,000- and 55,000-mol-wt polypeptides in BHK cells. (*A*, *C*, *E*, *G*, and *I*) show the rabbit anti-54/55 IF structural subunit staining patterns. (*B*, *D*, *F*, *H*, and *J*) show the monoclonal anti-IFAP-300K staining patterns. The two patterns and distributions were essentially coincident. (*A* and *B*) Early cell spreading after trypsinization and replating. Staining was concentrated in the juxtanuclear cap. (*C* and *D*) Spread cells 12 h after replating. Fibrous patterns were seen radiating outward from the cap. A slightly punctate pattern was noted in some cells with anti-IFAP-300K. (*E* and *F*) Fully spread cells 36 h after replating. The fluorescence was peripherally dispersed in a fibrous network. (*G* and *H*) Colchicine-treated cells. Both antibody patterns had retracted into the juxtanuclear cap, or ring. (*I* and *J*) 12 h after colchicine removal. Both antigens had redistributed to their normally dispersed, highly coincident patterns. (*A*, *B*, *E–J*) × 1,764. (*C* and *D*) × 1,120.


A
B
C

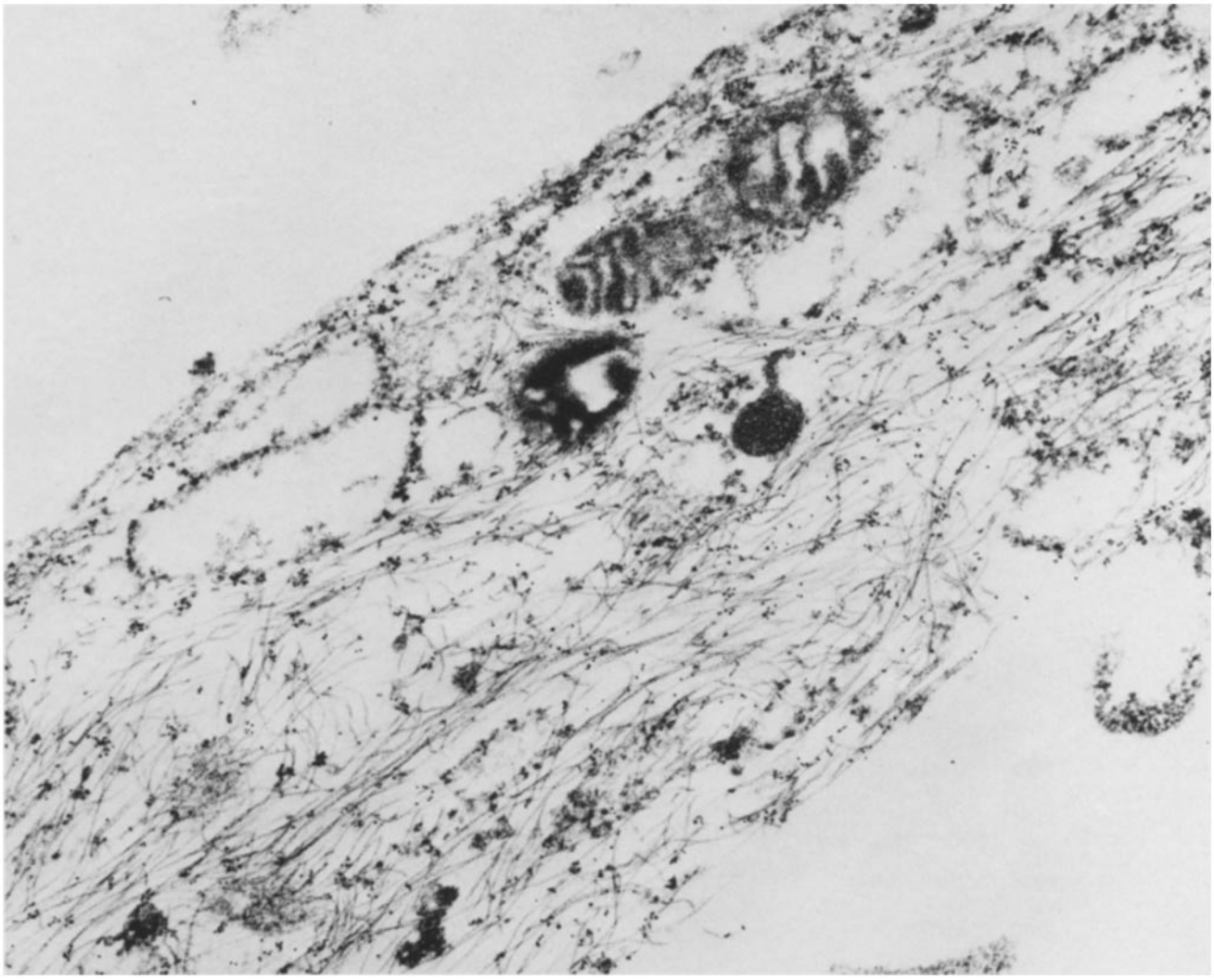


FIGURE 5 Association of IFAP-300K with dispersed IF networks. Indirect immunogold labeling of this cell process with anti-IFAP-300K demonstrated the apparently discontinuous distribution of IFAP-300K on or near IF and the closer association with IF, in a general sense, than with other cytoplasmic elements, such as mitochondria, microtubules, or membranes. The gold particles were often located at points on IF that were close to other IF. × 47,600.

approximated the IF pattern than it did any other cytoplasmic structural system, such as nuclei (Fig. 4*A*), mitochondria (Fig. 5), or microtubules (Fig. 7). Confidence in the specificity of the gold localization was obtained from the marked differences in pattern and intensity between IF and IFAP-300K labeling and by the essentially negative background deposition of gold particles observed when preimmune serum or control hybridoma culture supernatant was substituted for primary antibody (Fig. 4*C*).

Retention of IFAP-300K with IF after Disassembly/Assembly

Biochemical support for a specific association of IFAP-300K with BHK cell IF was provided by repeated cycles of disassembly/assembly of IF in vitro. As shown in Fig. 8*A*, the polypeptides solubilized by dialysis of a native IF preparation vs. 5 mM sodium phosphate (pH 7.1), 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride represented the structural

FIGURE 4 Specificity of in situ immunogold localization of IFAP-300K and 54/55 on IF. Following the protocol detailed in Materials and Methods, 5 nm colloidal gold-conjugated secondary antibodies were used to localize (*A*) monoclonal anti-IFAP-300K, (*B*) rabbit anti-54/55K, and (*C*) control hybridoma culture supernatant. Note in (*A*) the focal deposition of gold particles on IF, primarily at points of proximity between IF. A portion of the nucleus is visible at the left edge. In contrast, labeling of the structural subunits was essentially continuous along the lengths of individual IF (*B*). Note in the lower half of the field the long trail of gold particles apparently associated with no IF. This was due to the fact that these particles were localized to an IF that was out of the plane of the thin section. The control displayed minimal nonspecific labeling (*C*). Note in *C* the presence at many points of an amorphous material in the angles between some intersecting IF and at other points of proximity between IF. This may correspond to the material labeled in *A* by anti-IFAP-300K. × 78,400.

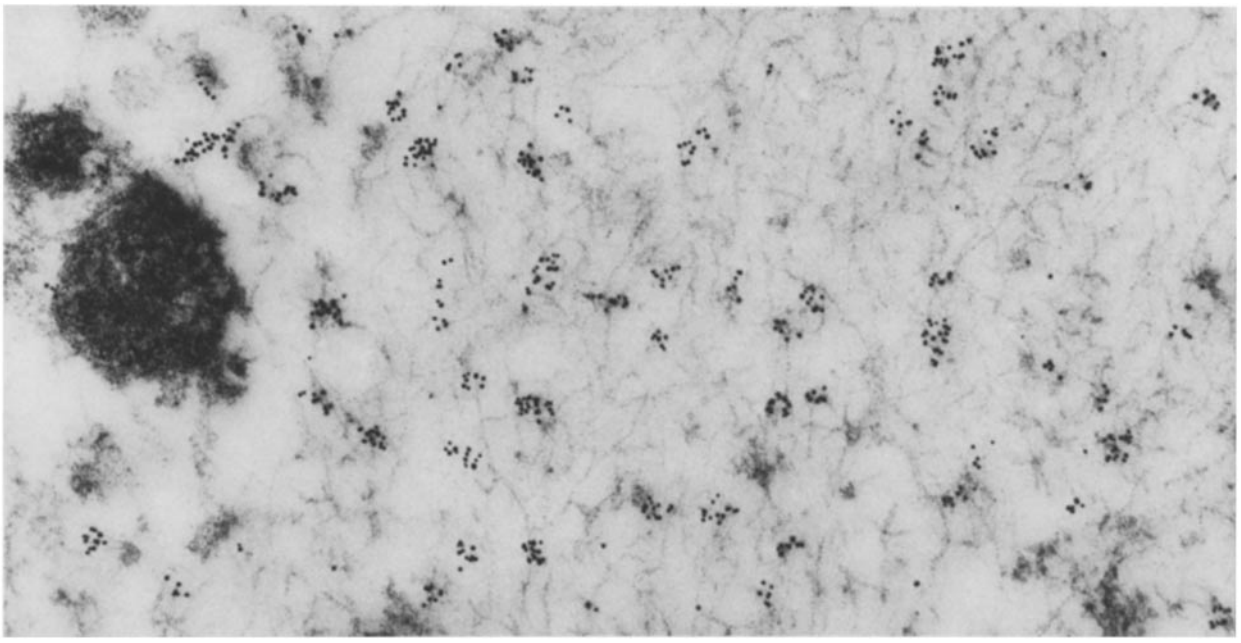


FIGURE 6 In situ immunogold localization of IFAP-300K in the juxtanuclear IF cap. In this characteristic, concentrated arrangement of IF, IFAP-300K remained associated with the IF in a focal pattern, usually at points where IF were close together. × 81,200.

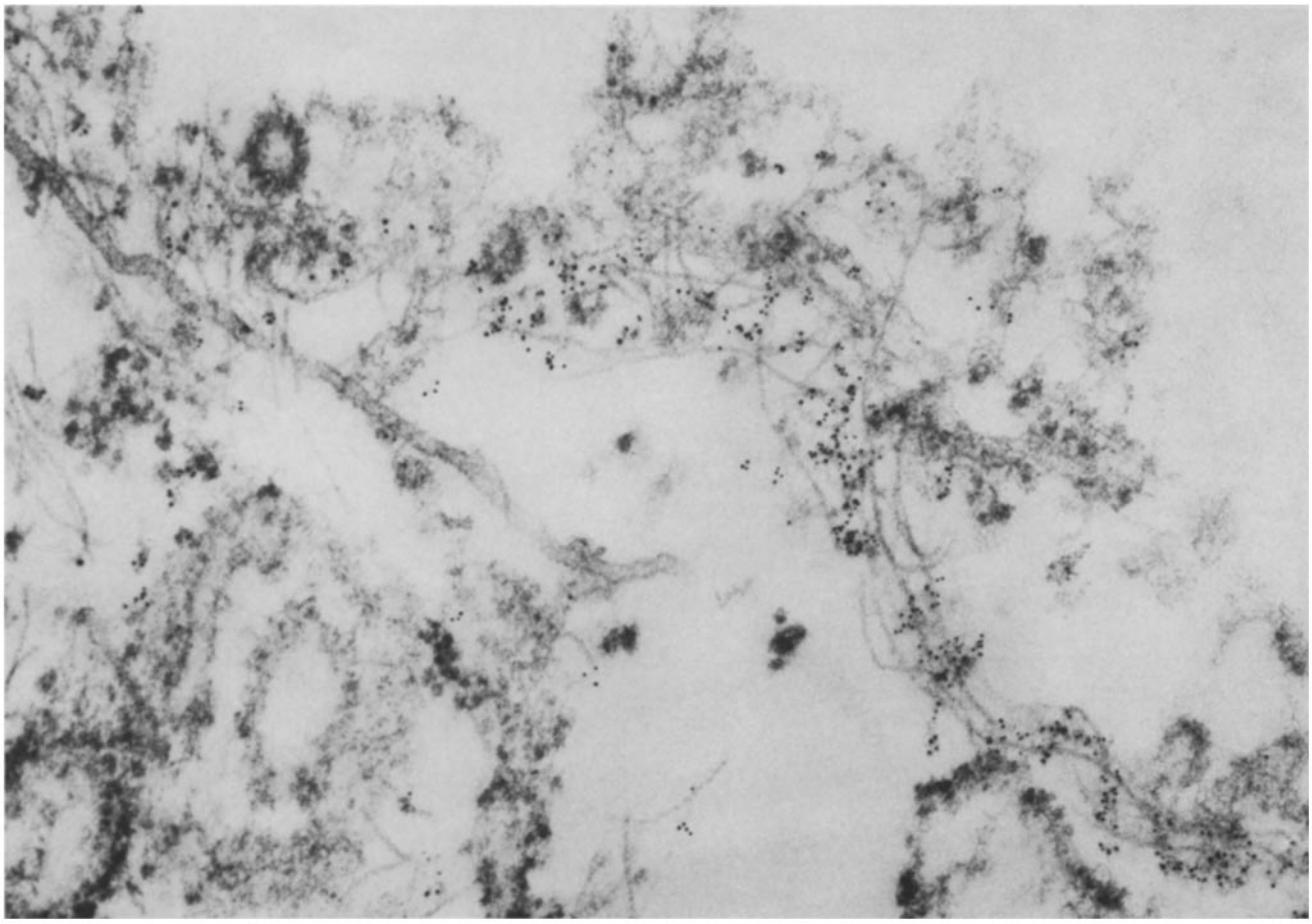


FIGURE 7 IFAP-300K associated with a bundle-like array of IF. IFAP-300K, detected by anti-IFAP-300K immunogold labeling, was apparently associated in a peripheral fashion with this IF array. × 98,000.

IF 54,000- and 55,000-mol-wt subunits as well as several minor constituents, including the 300,000-mol-wt species. In agreement with the earlier work of Zackroff and Goldman (54), ~40% of the total protein present in the IF preparation was extracted into this low salt buffer (S_1). The extraction was differential, however, in that the 60,000–70,000-mol-wt pro-

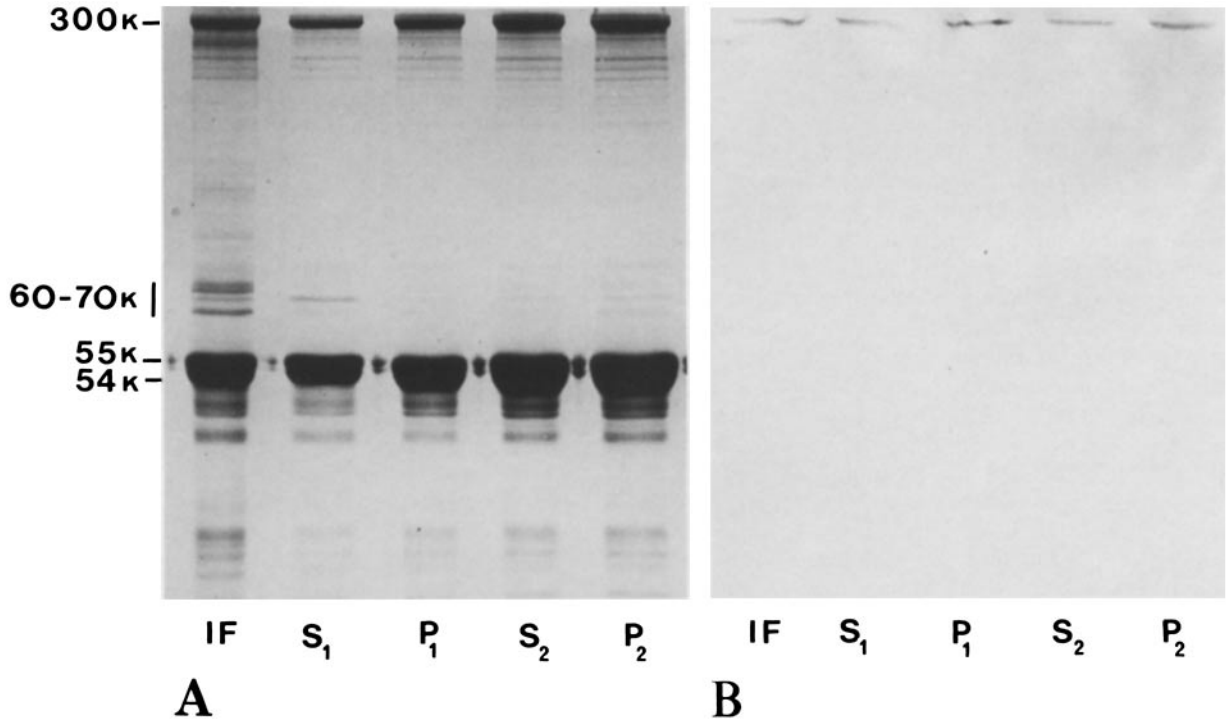


FIGURE 8 Co-disassembly/assembly of IFAP-300K/IF. We subjected native IF preparations to two cycles of disassembly/assembly to determine if the IFAP-300K co-fractionated with the IF 54,000- and 55,000-mol-wt structural subunits. (*A*) 7.5% polyacrylamide SDS gel of a native IF preparation in various stages of cycling. Coomassie Blue-stained. Molecular weights $\times 10^{-3}$ are indicated. *IF*, Native IF preparation; S_1, high-speed supernatant of low salt extract; P_1, pellet of once-cycled IF polymerized from S_1; S_2, low salt extract of P_1; P_2, pellet of twice-cycled IF polymerized from S_2. IFAP-300K was retained with the IF subunits. (*B*) Nitrocellulose transfer of *A* immuno-stained with anti-IFAP-300K confirmed co-cycling of this IFAP.

teins (the so-called keratinlike, paracrystal proteins [15, 55]) were only minimally represented, while the 300,000-mol-wt material persisted. After polymerization of IF from S_1 to yield the first cycle pellet (P_1), low salt extraction (5 mM sodium phosphate buffer) yielded an S_2 containing predominantly 54,000-, 55,000-, and 300,000-mol-wt subunit, a composition that was preserved in the IF polymerized from S_2 (i.e., P_2).

Immunoblots of SDS gels of the IF cycling sequence (Fig. 8 *B*) confirmed the retention of the 300,000-mol-wt species. The ability of IF and IFAP-300K to specifically reassociate from a disassembled state into a sedimentable complex provided in vitro biochemical support for the in situ morphological data described above. Taken altogether, these data were interpreted as reflective of a functional relationship between IFAP-300K and IF.

DISCUSSION

This study used a monoclonal antibody to show that a 300,000-mol-wt doublet polypeptide species present as a minor component in native BHK cell IF preparations was morphologically associated in situ with IF in the various organizational states assumed by the latter. This evidence was supported by the finding that the species retained its relationship with IF during in vitro disassembly/assembly cycling of IF. Earlier work has shown that such components are not necessary for the in vitro polymerization of IF (41).

Because the evidence strongly supports the possibility that the high molecular weight (300,000) protein described here represented an IFAP, and because there is evidence for the existence of other associated proteins in BHK cells (15, 53, 55), we propose that the acronym IFAPs (intermediate filament associated proteins) be used for the entire group of proteins whose association with cytoplasmic IF is supported by morphological, immunological and biochemical criteria. We proposed the descriptive term IFAP-300K for the associated protein of the present study. As there are no known functions for IFAPs, and there are probably many still to be identified, we feel that this nomenclature system is preferable to the use of specific names that often imply function. Such names have proliferated to the point of confusion in the recent cytoskeletal literature. Moreover, this terminology is in keeping with the nomenclature already developed for the microtubule-associated proteins (MAPs).

Because the use of monoclonal antibodies is especially advantageous in cases in which the specific antigens are difficult to identify or to purify in quantities sufficient for immunization, this technique is a powerful method for investigating the structure, intracellular location, and possible functions of IFAPs. Although the initial suggestion that the 300,000-mol-wt subunit may be an IFAP was the result of its very presence in the 0.6 M KCl/1% Triton cellular residue that contains the IF, the development and use of a monoclonal antibody probe was the main approach for establishing this contention. A hybridoma cell line that produced antibodies directed against the 300,000-mol-wt polypeptide was selected for this study.

The major point demonstrated by the use of this probe in immunofluorescence microscopy is that IFAP-300K morphologically approximates the cytoplasmic distribution and pattern of IF. Moreover, this association is a dynamic one inasmuch as the dramatic organizational changes that IF routinely undergo (e.g., from a dispersed network to a juxtanuclear cap and back to a network) are concurrently expressed

in the IFAP-300K fluorescence pattern. The fact that the IFAP-300K distribution parallels the IF distribution in all IF configurations studied in situ strongly suggests a specific association. The demonstration both immunologically and biochemically (M_r and peptide mapping) of the nonhomologous nature of IFAP-300K vs. 54,000 and 55,000-mol-wt subunits indicates that the immunofluorescence findings are not the result of the two antibodies' labeling different antigenic determinants on the same proteins, namely the 54,000- and 55,000-mol-wt proteins.

The morphological association implied by immunofluorescence between IFAP-300K and IF is confirmed at the ultrastructural level using immunogold labeling. For this purpose, a fixation protocol that preserves antigenic determinants on IFAP-300K and 54,000- and 55,000-mol-wt subunits has been devised. This protocol also preserves cell shape, adhesion to growth substrates, and the overall morphology of various organelles, including mitochondria, nuclei, microfilaments, and microtubules. The latter consideration is important in determining that IFAP-300K is associated morphologically with the IF cytoskeletal system in its various organizational states, as opposed to other filament systems or organelles. Gold label is occassionally found in association with other cellular constituents, but IF are usually nearby. The problem of nonspecific labeling due to binding by the colloidal gold moiety itself instead of by the specific antibody appears to be minimal in this study based upon the low background obtained when preimmune serum or control hybridoma culture supernatant are substituted for the primary antibodies (Fig. 4*C*). Perhaps even more indicative of the validity of antigen localization are the obvious differences in gold label patterns and distributions between anti–IFAP-300K and anti-54/55.

The anti–IFAP-300K gold label observations raise many interesting possibilities as to the function(s) of IFAP-300K. The nature of the association of the molecule with IF may indicate a role in linking the individual filaments into a three-dimensional lattice. This role could be especially important in maintaining the extensive, apparently interconnected system seen in fully spread cells. The gold particles seen extending outward from IF and not associated with any apparent structure may be the result of their being connected to IF that are located out of the plane of the thin section, leaving only a "trail" of label to be seen. However, such extensions may represent 300,000-mol-wt–containing "bridges" between IF, or between IF and other structures. Finally, the detection of IFAP-300K label in association with loose, bundlelike arrays of IF (see Fig. 7) suggests a role in forming or maintaining such arrangements.

IFAP-300K appears to be distinct from other high molecular weight IFAPs that have been described. One of these is a family of cationic, keratin-associated proteins termed the filaggrins (5, 39). These proteins are involved in cross-linking keratin-containing IF into tightly packed bundles, or macrofibrils (39). Recent evidence suggests that the physiologically active form of the (~30,000-mol-wt) filaggrin polypeptide is derived from a high molecular weight (400,000) precursor molecule (8, 40). While these proteins are capable of interacting in vitro with subclasses of IF other than keratin, they have been described in situ only in cells possessing keratin IF. Moreover, as a group they differ markedly from IFAP-300K with respect to the M_r of the active form of filaggrin (~30,000), amino acid composition (extremely high Ser, Gly, His, and Arg and negligible Lys and Leu [5, 39] relative to IFAP-300K [see Table I]) and apparent function (organizes IF only into tight parallel arrays to form microfibrils [6, 39]).

With respect to high molecular weight IFAPs, synemin, a 230,000-mol-wt species originally isolated from chicken gizzard muscle (17), has been shown by immunofluorescence criteria to co-localize with both vimentin and desmin IF in a variety of cell types (17, 19). Comparison of the amino acid composition of this protein (35) with that of IFAP-300K reveals some similarities in overall acidic and basic residue content, but rather marked variation for Ala, Val, Ile, and Leu. Moreover, the in-register immunolocalization of synemin on adjacent IF suggests a lateral IF-IF linking function for this protein that appears to be spaced at regular intervals along IF (18). Based upon these parameters, it would seem that IFAP-300K is distinct from synemin.

The high molecular weight protein, paranemin, also appears to interact with vimentin and desmin IF (2, 32). This 280,000-mol-wt protein is present in cultured embryonic avian skeletal muscle, but not in adult muscle. It is also found by immunofluorescence in a subpopulation of cultured chicken embryonic fibroblasts in association with vimentin IF. Together with synemin, paranemin has a complex developmental association pattern with desmin/vimentin, primarily in cardiac and smooth muscle cells, but also in some nonmuscle cell types (32). While the M_r and the property of co-distribution with desmin/vimentin are suggestive of paranemin, the pI and epithelial cell distribution pattern of IFAP-300K (unpublished observations) are markedly different from those of paranemin, supporting their nonidentity.

Plectin is a 300,000-mol-wt protein originally identified in IF-enriched preparations from glioma cell cultures (33). This protein has been described as a general "cytoplasmic element cross-linker" (49), as well as a possible factor involved in the formation of cell junctions (52). These conclusions are based upon several observations including the following: (*a*) the similarity between plectin and high molecular weight microtubule-associated proteins (MAPs) (33, 50); (*b*) plectin's co-distribution with vimentin-containing IF in colcemid-treated cells as determined by immunofluorescence (49), as well as its proposed biochemical association with vimentin (51); and (*c*) the wide spectrum of cellular specializations (e.g., desmosomes and other junctional complexes, muscle Z-lines, cardiac intercalated disks) stained by indirect immunofluorescence using antiserum directed against plectin (52). In contrast, our preliminary results in comparing MAPs and IFAP-300K using SDS PAGE/immunoblotting with anti–IFAP-300K and anti-MAPs or antitubulin show no cross-reactivity. In addition, no staining of desmosomes in epithelial cells has been seen with our antibody (unpublished observations).

In summary, our results suggest that in situ IFAP-300K is predominantly associated with IF in a discontinuous manner, especially at points of proximity between IF. This association is seen to be maintained for the various IF configurations representative of different physiological and experimental cell states. Moreover, this association can be reestablished after in vitro disassembly of the IFAP-300K/IF complex. Until more information is forthcoming, it would appear that IFAP-300K is distinct from the other high molecular weight IFAPs reported to date and therefore represents a newly described protein.

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