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.
characterized with respect to their polypeptide staining pattern on polyacrylamide gels (38). The major species possess 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% CO2. 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, 6 mM Na**K phosphate [pH 7.4], 17 mM NaCl, 3 mM KCl). 10 ml of cold lysis buffer (0.6 M KCI, 10 mM MgCl2, 1% Triton X-100 in PBS) 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, 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 each 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 centrifuged at 26,000 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 S0. 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 (P1) represented once-cycled IF. The procedure was repeated to yield twice-cycled IF (P2).

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-
emulsified in an equal volume of Freund's complete adjuvant (Gibco Labora-
tories) 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 immunoblots using the procedure of Towbin et al. (44). 24 hours 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 pro-
cedures (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 hybridomas 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 immu-
noblotting.
Identification of Monoclonal Antibody Subclass: The anti-
bodies present in the culture supernatants of the hybridoma cell lines were
determined according to the method of Rammensee et al. (57). Briefly, IFAP-300K
was solubilized (5 mM Tris-HCl [pH 7.4]) and IF preparations were coated on micro-
plates and incubated successively with culture supernatants, rabbit anti-mouse
antibody, and peroxidase-conjugated goat anti-rabbit IgG. The reactions were
visualized by 2.2-azino-di-[3-ethyl-benzthiazoline sulfonate]/H₂O₂ solution as
quantiﬁed by measuring the optical density of each well at 415 nm.

Immunoblotting: After SDS PAGE of solubilized IF, one sample lane
decided by the technique 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 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 results of the control
of nitrocellulose were then incubated in a solution of 10% fetal calf serum, 2.5%
bovine serum albumin in PBS, for 1 h, rinsed briefly in PBS, and then cut
into strips that were incubated overnight with the monoclonal antibodies or a
bovine serum albumin in PBS~ for 1 h, rinsed briefly in PBS, and then cut
into strips that were incubated overnight with the monoclonal antibodies or a
bovine serum albumin in PBS~ for 1 h.

Secondary Absorption with Antigen: 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 in-
cluded the preabsorption of antibody preparations with antigen. These were
performed on anti-IFAP-300K by incubating monoclonal culture supernatants
with microcellulose 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 IJIRS
epifluorescence system (Carl Zeiss, Inc., Thornwood, NY). Fluorescence micro-
graphs were taken with Kodak Plus-X film (Eastman, 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, for 30 min, washed in PBS, and postfixed in 1%
OsO₄ in PBS, 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 Immunoglobulin Localization: For the ultrastruc-
tural localization of IFAP-300K by the immunogold technique (7), we prepared
BHk-21 cells grown in 35-mm culture dishes by the following protocol using a 2-(N-morpholino)ethane sulfonic (MES) acid (Research Organ-
ical, OH) buffer system: (a) Three rapid washes in PBS. (b) Fixation for 15–
30 min in 0.07-0.1% glutaraldehyde, 0.15% Triton X-100 in MES buffer (0.1 M
MES [pH 6.6]. 0.5 mM MgSO₄, 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 NaBH₄ in MES buffer for 20 min
to reduce free aldehyde groups on glutaraldehyde that can nonspecifically bind
collodial 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
30 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 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, em-
bedded 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 re-
presented 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. 1A). A group of related poly-
petides (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 mul-
tiple 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 x 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.

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 IgG2a 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. 1A). The monoclonal antibody reacted exclusively with this doublet on immunoblots of these gels (Fig. 1D). Rabbit antiserum directed against the 54,000- and 55,000-mol-wt structural subunit proteins did not cross-react with IFAP-300K (Fig. 1C). 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 occasionally revealed a faint reaction between anti-IFAP-300K and a 250,000-mol-wt band (Fig. 1D).

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 x, 3 x, and 5 x 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.

...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.
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.

By the same criteria, the 250,000-mol-wt protein was closely related to IFAP-300K and probably represented a proteolytic 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 associated with IF (Figs. 4A 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. 4A and 5). It should be noted that in some preparations (e.g., in Fig. 5), the 54,000-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. 4A, 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 structural IF 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

### Table I

|       | 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 hydrolysates for each protein. Cys and Trp values were not determined.

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. 4A and 5), thus corroborating the co-distribution of the two antigens as detected by immunofluorescence (Fig. 3). More specifically, comparison of Fig. 4A 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. 4A 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. 4A, 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. 4A, 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 structural 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.
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. 4A), 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. 4C).

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. 8A, 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.
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 (S1). The extraction was differential, however, in that the 60,000–70,000-mol-wt pro-
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. 8B) 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 (Mr 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, associated morphologically with the IF cytoskeletal system in its various organizational states, as opposed to other filament systems or organelles. Gold label is occasionally 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 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 microfibrils (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 Mr 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 Mr and the property of co-distribution with desmin/vimentin are suggestive of paranemin, the pl 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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