Purification of the 300K Intermediate Filament–associated Protein and Its In Vitro Recombination with Intermediate Filaments

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ABSTRACT IFAP-300K is a 300,000-mol-wt intermediate filament–associated protein previously identified in the baby hamster kidney fibroblastic cell line (BHK-21) by a monoclonal antibody (Yang H.-Y., N. Lieska, A. E. Goldman, and R. D. Goldman, 1985, J. Cell Biol., 100: 620–631). In the present study, this molecule was purified from the high salt/detergent-insoluble cytoskeletal preparation of these cells. Gel filtration on Sephacryl S-400 in the presence of 7.2 M urea allowed separation of the high molecular weight fraction from the structural intermediate filament (IF) subunits desmin and vimentin, designated 54K and 55K, respectively, and other low molecular weight polypeptides. DE-52 cellulose chromatography of the high molecular weight fraction using a linear NaCl gradient in 8 M urea yielded a pure 300,000-mol-wt species which was confirmed to be IFAP-300K by immunological and peptide mapping criteria. Two-dimensional PAGE of native BHK IF preparations followed by immunoblot analysis demonstrated the inability of the IFAP-300K–immunoreactive material to enter the first dimensional gel except as a 200,000-mol-wt doublet which presumably represented a major proteolytic derivative of IFAP-300K. The molecule's pI of 5.35, as determined by chromatofocusing, and its amino acid composition were extremely similar to those of BHK cell vimentin/desmin despite their non-identity. Ultrastructurally, IFAP-300K preparations in low salt buffers existed as particles composed of one or two elliptical units measuring 16 × 20 nm. In physiological salt buffers, the predominant entities were large, elongated aggregates of the elliptical units, which were able to be decorated by using the immunogold technique with monoclonal anti-IFAP-300K.

Compared with the morphology of homopolymer vimentin IF, in vitro recombination studies using column-purified vimentin and IFAP-300K demonstrated the additional presence of aggregates similar in appearance to IFAP-300K at points of contact between IFs. Antibody decoration and immunogold labeling of these recombined preparations using rabbit anti-desmin/vimentin and monoclonal anti-IFAP-300K confirmed the identity of the inter-filament, amorphous material as IFAP-300K. The presence of IFAP-300K at many points of intersection and lateral contact between IFs, as well as at apparent inter-filament "bridges," in these recombined specimens was identical to that seen both in situ and in native IF preparations. These results were supported biochemically by the co-sedimentation of recombined IFAP-300K and vimentin IFs to yield complexes whose compositional stoichiometry was similar to that observed for these proteins in native IF preparations. No such co-sedimentation was found in vitro between actin and IFAP-300K. No effects of IFAP-300K upon the kinetics of IF polymerization were detected by turbidimetric measurements.

Intermediate filaments (IFs)[1] are a class of fibrous, cytoplasmic filaments whose intracellular and tissue distribution have been described by various biochemical, morphological, and immunological criteria (19, 20, 32, 43). The classification of IFs into subgroups has also been suggested on the basis of these criteria (7, 12, 19, 20, 24). Though it is very useful, this classification has not sharply defined IFs as to cell type distribution as originally proposed, due to the identification of more than one class of IF (6, 9, 10) in a given cell type and due also to the fact that some cell types contain an IF subunit class which is not representative of the cell's embryological origin and morphology (8, 26, 34, 35).

Despite the diversity of the classes of IF with regard to the number of major subunit proteins, and their molecular weights, isoelectric points, and solubility properties, most types have the ability to self-assemble in vitro (19, 20, 43). However, the conditions used for in vitro disassembly/assembly of IFs can only be remotely reflective of respective in vivo regulatory mechanisms, especially with regard to disassembly. Furthermore, the spectrum of supramolecular organizations which IF networks can assume in various cell types is apparently not determined directly by the major IF structural subunits themselves. In this regard, the numerous, minor polypeptides present in IF-enriched cytoskeletal preparations from various cell types may be significant. Several of these proteins have been identified, and it has been suggested that some are IF-associated proteins (IFAPs). These IFAPs show varying degrees of expression among different tissue types (for example, epinemin [17], p50 [36], paranemin [25], plectin [38], and synemin [14]). However, the only group of IFAPs for which there is any direct evidence regarding possible function is the filaggrins (5, 31). This family of basic, keratin-associated proteins which are found in epidermal cells appears to have a role in regulating IF bundle formation. Keratin IFs reassembled in the absence of filaggrin display a random configuration of criss-crossing filaments. In vitro, filaggrin induces the bundling of such filaments into parallel arrays of macrofibrils which are analogous to the tonofilaments seen in epithelial cells such as keratinocytes (31).

Using a monoclonal antibody approach, we have recently been able to identify a 300,000-mol-wt polypeptide in baby hamster kidney (BHK-21) cell IF preparations which, by immunofluorescence criteria, co-distributes with IFs in situ. Moreover, this species partitions with vimentin/desmin during disassembly/assembly cycling of BHK-21 IF preparations and, on the basis of ultrastructural immunogold labeling, is localized on IFs most frequently at points of proximity between IFs (40). This protein, which we have designated IFAP-300K, may function in determining organizational states of IF, as well as their intracellular distribution. To gain more insight into the properties and functions of IFAP-300K and to determine more precisely its mode of interaction with IF, we present methods for the biochemical purification of IFAP-300K from BHK-21 cells. We then preliminarily characterize the purified protein and demonstrate in vitro its capacity to recombine with purified homopolymer IFs in the same arrangement as observed in situ.

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[1] Abbreviations used in this paper: anti-54/55, rabbit antiserum against the 54K and 55K BHK IF structural proteins; DTT, dithiothreitol; IFs, intermediate filaments; IFAPs, IF-associated proteins; IFAP-300K, a 300,000-mol-wt IFAP in BHK-21 cells.

MATERIALS AND METHODS

Cell Culture: Baby hamster kidney cells (BHK-21/C13) were grown as stock cultures on 100-mm plastic culture dishes as previously described (40). Carbon dioxide–purged roller bottle cultures were incubated at 37°C to obtain preparative quantities of cells.

Isolation of IFs: IFs were isolated by the procedure of Zackroff and Goldman (42) as modified from Starger et al. (30). Protein concentrations were determined by absorbance at 280 nm using salt-free, lyophilized BHK IF as a standard.

IFAP-300K Purification: A pellet of native BHK IF (1 g wet weight) was prepared from 30 confluent roller bottle cultures. This was homogenized in 18 ml of 7.2 M urea (Schwarz/Mann Div., Cambridge, MA), 50 mM Tris-HCl, pH 7.4, 4 mM EDTA, 2 mM EGTA (Sigma Chemical Co., St. Louis, MO), 2% ethylene glycol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% n-tosyl-L-argine methyl ester, and solubilized by dialysis versus two changes of 500 ml each of the buffer for 12 h at 4°C. The material was centrifuged in a Beckman JS-13 swinging bucket rotor at 26,000 g for 20 min at 4°C followed by centrifugation at 105,000 g for 30 min at 10°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). The supernatant (~20 ml) was applied to a 2.6 × 90-cm column (480 ml) of Sephacryl S-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the same buffer. Filtration was allowed to proceed at a flow rate of 26.5 ml/h with tube fractions of 7.8 ml being collected. The eluant was monitored by absorbance at 280-nm wavelength. The tube fractions containing IFAP-300K were identified by SDS PAGE and immunoblotting (see below) using a monoclonal antibody (anti-IFAP-300K) which has been described previously (40).

The respective pooled tube fractions (~40 ml) were dialyzed versus 500 ml (two changes at 4°C for 18 h) of 8 M urea, 50 mM Tris-HCl, pH 8.2, 1 mM EDTA, 1 mM dithiothreitol (DTT) (Sigma Chemical Co.), and 2% ethylene glycol. The fraction was then applied to a 1 × 35-cm column (27 ml) of DE-52 (diethyl aminoethyl) cellulose (Whatman Ltd., Kent, England). Following release of unbound material under starting buffer conditions, a linear gradient of 0–45 mM NaCl (98 ml each in starting buffer) was developed at a flow rate of 16 ml/h. The eluant was monitored by absorbance at a wavelength of 280 nm, and 5-ml fractions were collected. Upon completion of the gradient, residual protein was released from the exchanger by passage of two column bed volumes of 1 M NaCl in starting buffer. The fractions were assessed by SDS PAGE and immunoblotting using anti-IFAP-300K. Storage was at 4°C.

Vimentin Purification: Vimentin-containing tube fractions from the S-400 gel filtration step above were identified by SDS PAGE. Vimentin, designated 55K and referred to as this herein, was purified from this fraction by the ammonium sulfate precipitation/DE-52 chromatographic technique of Lieska et al. (21) with only slight modification. The most concentrated fractions were pooled (~30 ml) and subjected to ammonium sulfate fractionation by the addition of solid NaCl to 525 mM and solid $(NH_4)_2SO_4$ to 35% saturation (disregarding the presence of 8 M urea in sample). Following precipitation at 4°C for 1 h with stirring, the specimen was centrifuged at 20,000 g for 25 min at 4°C. The resultant pellet was dispersed in 5 ml of the above DE-52 column buffer, except that 15 mM NaCl was included and ethylene glycol was omitted. The specimen was dialyzed versus the same. After chromatography under the same conditions as for IFAP-300K purification (except that a 15–45-mM NaCl gradient was used), the tube fractions containing pure 55K were identified by SDS PAGE and immunoblotting (see below).

Chromatofocusing: Chromatofocusing of IFAP-300K–containing fractions was performed from pH 7.4 to pH 4.0 on a 1 × 20.5-cm column of Polybuffer Exchanger 94 (Pharmacia Fine Chemicals). The start buffer, in which the sample and the exchanger were equilibrated, consisted of 8 M urea, 1 mM EDTA, 1 mM DTT, and 50 mM Tris-HCl, pH 7.4. After sample loading, 200 ml of 8 M urea, 1 mM EDTA, 1 mM DTT, Polybuffer 74 (diluted 1:8; adjusted to pH 4.0 with HCl) was passed at a flow rate of 28 ml/h to develop the internal pH gradient. The eluant was monitored by absorbance at 280-nm wavelength, and the pH of alternate 2.6-ml fractions was measured.

PAGE: Analytical SDS PAGE was performed according to Laemmli (16) on 7.5% polyacrylamide slab separating gels with 4.5% stacking gels. Two-dimensional gel analyses were performed by the method of O'Farrell (23) using several different pH ranges. In some instances denaturants additional to 9 M urea were included in two-dimensional sample preparations and/or the focusing gel. These included 1–10% non-ionic detergents (Nonidet P-40, Triton X-100), glycerol, ethylene glycol, 5 mM EDTA, and 5% β-mercaptoethanol (or 5 mM DTT).

All gels were stained with 0.1% Coomassie Blue R-250. The distribution of protein among the resolved bands was determined by densitometric scanning of the SDS gels at 570-nm wavelength.

Amino Acid Analysis: Purified column fractions were dialyzed

against 1 mM NH₄HCO₃ and lyophilized before hydrolysis of ~3 nmol each with 6 N HCl at 108°C for 24 h. The dried hydrolysate was analyzed in a JEOL 6AH amino acid analyzer.

Immunological Techniques: A monoclonal antibody (15, 28) of the IgG₂ᵦ subclass directed against IFAP-300K has been previously produced and characterized (40). To obtain monoclonal antibody preparations of higher titer than obtainable by in vitro hybridoma culture, the cells were grown in the peritoneal cavities of BALB/C mice. This was accomplished by injection of 2 × 10⁶ hybridoma cells (in culture medium) intraperitoneally into each mouse. The resultant ascites fluid was withdrawn and monitored for anti-IFAP-300K activity by immunofluorescence microscopy and immunoblotting as previously described (40). A polyclonal rabbit antiserum against both the desmin (54K) and the 55K structural subunits of BHK cell IFs has also been described elsewhere (40).

Immunoblotting was performed by the method of Towbin et al. (33) and has been described elsewhere (40). SDS (0.1%) was included in the transfer buffer to enhance transfer of high molecular proteins.

Electron Microscopy: Specimens for negative staining, prepared on carbon-stabilized, formvar-coated 300 mesh grids, were stained with 3% uranyl acetate and examined in a JEOL 100CX electron microscope. Thin sections of IF pellets were prepared and examined as previously described (40).

Ultrastructural Localization Using Antibody Decoration and Immunogold Labeling: 500 μl of 6 mM Na⁺-K⁺ phosphate, pH 7.4, 170 mM NaCl, 3 mM KCl (PBSₐ) containing dispersed IFs (native IF preparations or various types of in vitro polymerized IFs) at ~200 μl/ml were incubated with 50 μl of monoclonal anti-IFAP-300K (ascites fluid) at room temperature for 40 min. The mixture was layered onto 0.5 ml of 2 M sucrose/PBSₐ (NaCl concentration adjusted to 171 mM) and centrifuged at 100,000 g for 1 h at 20°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc.). This allowed removal of unreacted antibody (in supernatant) from antibody-labeled IFs, which formed a broad layer in the sucrose and were recoverable in a dispersed form. The IF layer was dispersed in 500 μl of PBSₐ with a pipette and negatively stained for monitoring antibody decoration. For immunogold labeling, the dispersed IF layer was further incubated with 50 μl of 5 nm gold conjugated–goat anti-mouse IgG/IgM (Janssen Pharmaceutica, Brussels, Belgium) at room temperature for 40 min. This mixture was layered onto a 0.5-ml cushion of 2 M sucrose/PBSₐ and centrifuged at 70,000 g for 30 min at 20°C. A pink-colored zone (indicating the presence of gold label) developed in the center of the cushion. This zone was removed, dispersed in 200 μl of PBSₐ, and the suspension examined by negative staining. For thin section analysis, the zone was diluted with 500 μl of PBSₐ and the gold-labeled IFs were pelleted by centrifugation at 100,000 g for 30 min at 20°C.

Control experiments consisted of substitution of anti-IFAP-300K with a nonreactive hybridoma culture supernatant or with anti-54/55, a rabbit antiserum against the 54K and 55K BHK IF structural proteins (40).

In Vitro Recombination of IFAP-300K and 55K IFs: The capacity of purified 300K to recombine with homopolymer IFs polymerized from purified 55K, as well as its localization within such a complex, was investigated by the immunogold technique and by co-sedimentation studies. The DE-52 column fraction containing purified 55K was dialyzed at 4°C from the urea column buffer into low salt buffer (5 mM Na⁺-phosphate, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) to generate the soluble, protofilamentous state of IFs (as defined by Zackroff and Goldman [42]). The 300K fraction was similarly processed. The fractions were clarified by centrifugation at 131,000 g for 1 h at 4°C. The supernatants were then combined in various weight ratios, based upon A₂₈₀ determinations (55K concentration, ~200μg/ml), and the mixtures dialyzed versus 100 vol PBSₐ at room temperature for 16 h (two changes) to polymerize IFs. Alternatively, the addition of one-tenth vol of 1.87 M NaCl in low salt buffer was used to effect polymerization over a 3-h period. The mixtures were centrifuged at 100,000 g for 45 min at 23°C to collect as a pellet the IFs and any bound 300K. 55K in low salt buffer also was polymerized into IF in the absence of 300K, followed by the addition of 300K to the pre-formed filaments. These mixtures were then incubated for 4–6 h before centrifugation at 100,000 g for 45 min at 23°C. Controls consisted of the parallel preparation of dialyzed fractions containing either 55K or IFAP-300K alone. Aliquots were taken at each stage from control and recombined specimens for analysis by SDS PAGE. All samples were further examined by negative stain electron microscopy before and after centrifugation. Recombination specimens were also examined morphologically following incubation with monoclonal anti-IFAP-300K or with anti-54/55. Recombination specimens incubated with anti-IFAP-300K were also examined following subsequent treatment with gold-conjugated secondary antibody.

As a control for the interaction of IFAP-300K with a cytoplasmic filament type other than the IF, co-polymerization with actin was investigated. Actin was prepared from acetone powder of chicken breast muscle by the method of

Spudich and Watt (29). Combination with IFAP-300K was performed at 4°C by effecting G → F transformation of the actin in these mixtures (1:10 ratio) by the addition of one-tenth vol 1.87 M NaCl in low salt buffer. Excess CaCl₂ and ATP (Type II; Sigma Chemical Co.) in the G-actin solution had been removed by 4 h dialysis versus low salt buffer. Specimens were centrifuged at 80,000 g for 2 h at 4°C, and the pellets and supernatants analyzed by SDS PAGE.

Turbidimetric Measurements: The kinetics of IF polymerization in the presence and absence of IFAP-300K were monitored by time-course turbidimetry for 1–16 h at a wavelength of 340 nm at 23°C (42) using a Beckman model DU-7 spectrophotometer and kinetics accessory unit (Beckman Instruments, Inc.).

RESULTS

Immunogold Localization of IFAP-300K in Native IF Preparations

It was previously shown by SDS PAGE and immunoblot analysis (40) that IFAP-300K was present in native IF preparations from BHK cells. To morphologically investigate the association of IFAP-300K with IF in the in vitro state, the molecule was localized in IF preparations using the indirect immunogold technique. In such preparations, the molecule was distributed in a fashion essentially identical to that observed in situ (40), namely in a discontinuous pattern along IF at points of proximity or contact with other IFs (Fig. 1 *A*). The IF-associated element on which the gold was deposited was often visible as amorphous, proteinaceous material tightly adherent to filaments at these points (Fig. 1 *B*). These results morphologically supported the previous evidence (40) that 300K was an IFAP which could be co-isolated with IF by virtue of its physical association with them.

Purification of IFAP-300K

The initial objective was to separate the high molecular weight polypeptides from the IF structural subunit proteins desmin and vimentin, designated 54K and 55K, respectively, which constitute ~80% of the total protein in native BHK IF preparations (based upon SDS PAGE analysis), as well as from the 60–70K so-called keratin-like polypeptides (41) and the 40–45K region material. Gel filtration on Sephacryl S-400 in the presence of 7.2 M urea proved most effective (Fig. 2). By SDS gel analysis, only those fractions most concentrated in 300K and least contaminated by the other species were pooled for further purification. The SDS PAGE pattern of this pool was identical to that of the corresponding region in SDS gels of native IF preparations. The yield of 300K was ~80% at this stage.

Chromatography of this pool on DE-52 cellulose generated two fractions, of which the one eluted by 30 mM NaCl in the gradient contained only the 300K doublet polypeptides (Fig. 3*A*). A significant quantity (~50%) of the protein applied to the column apparently was irreversibly bound to the DE exchanger. Only minimal protein could be eluted after the passage of the salt gradient, even using 1 M NaCl in starting buffer adjusted as low as pH 3.0. This reduction appeared to equally affect both fractions separated on this column. The combined yield of 300K from these procedures was ~40%, or 1.2 mg protein.

Purification of 55K

DE-52 cellulose chromatography of 55K-enriched fractions from the gel filtration step above yielded a 55K fraction
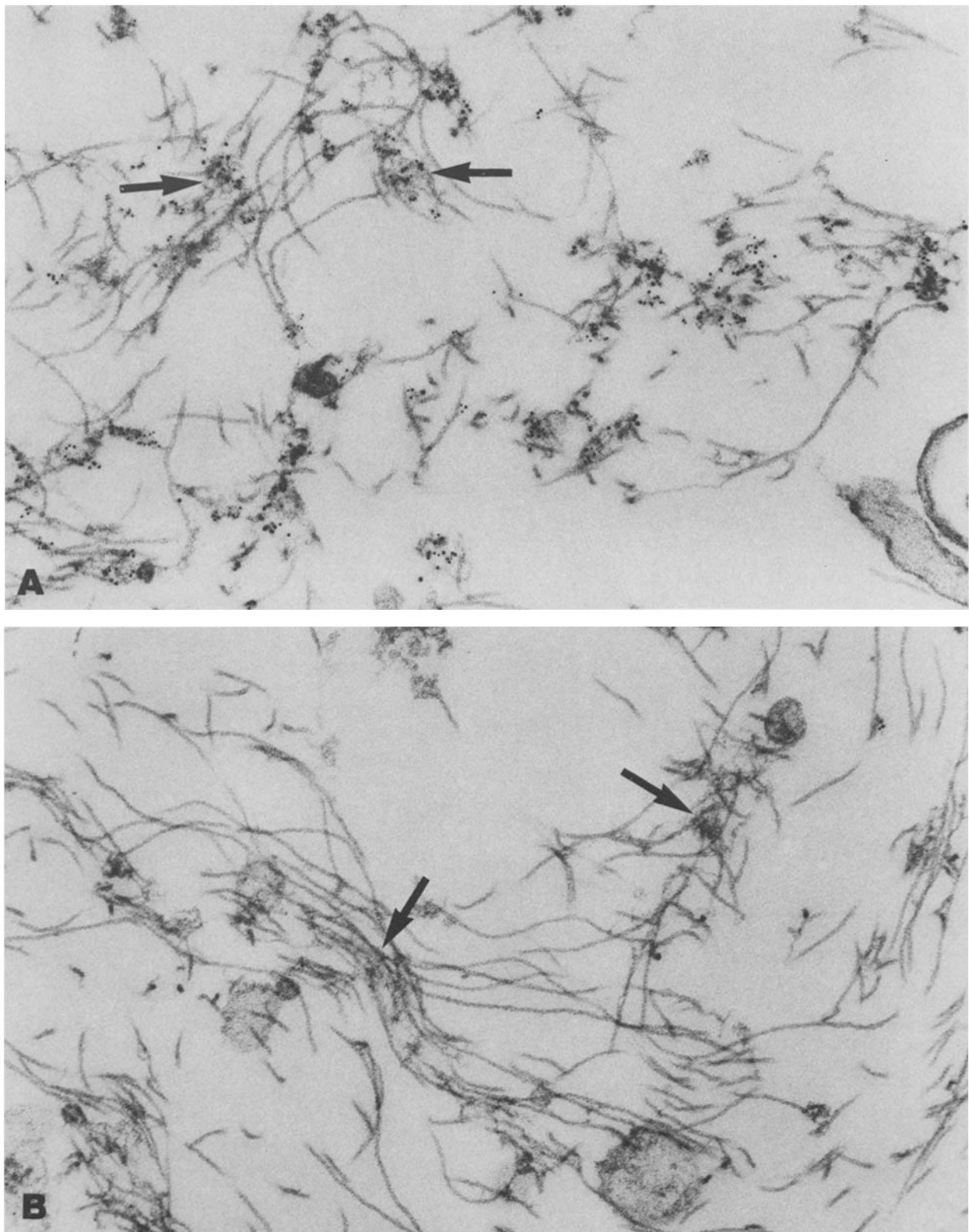
FIGURE 1   Localization of IFAP-300K in native BHK cell IF preparation. (A) Immunogold localization using monoclonal anti-IFAP-300K yielded a pattern of gold particles focally distributed along the IFs, primarily associated with an amorphous material (arrows), at points of proximity or contact between individual IFs. An identical distribution was seen in situ in BHK cells (37). (B) Control: unreactive hybridoma culture supernatant was substituted for anti-IFAP-300K. The background deposition of gold was essentially non-existent. The amorphous material between IFs (arrows) occupied the corresponding location of the gold-localized elements in A. Embedded and sectioned preparations. × 94,000.

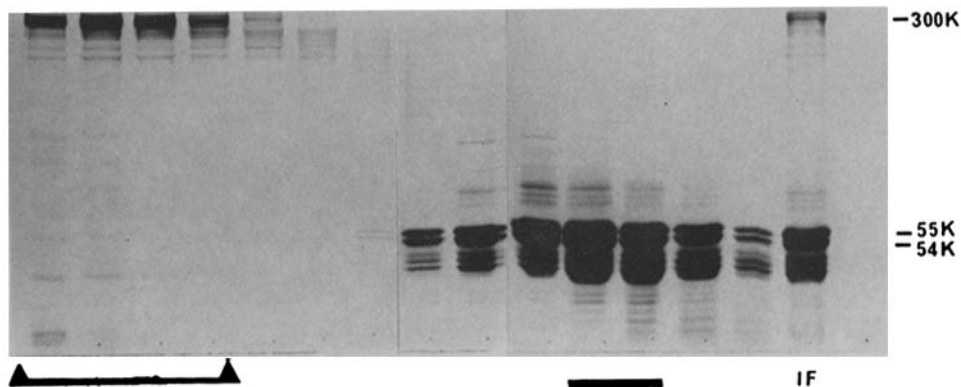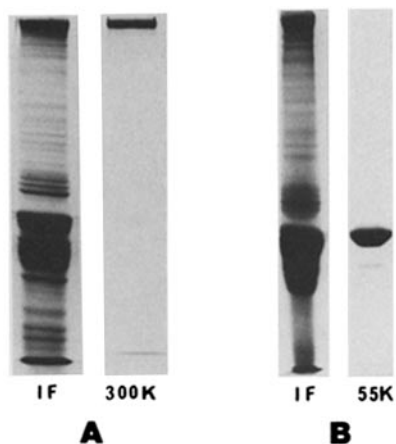FIGURE 3 Purification of BHK cell 300K polypeptide and 55K. (A) DE-52 cellulose chromatography of the high molecular weight fraction from the gel filtration step (▲——▲ in Fig. 2) yielded purified 300K, as shown by SDS PAGE. IF, marker lane of native IF preparation. (B) DE-52 cellulose chromatography of the 35% ammonium sulfate pellet of the 55K-containing fractions from the gel filtration step (—— in Fig. 2) allowed purification of 55K.

(combined yield ~50%, thereby comparing favorably with those of Lieska et al. [21] and Geisler and Weber [11]) containing only a trace amount of ~40K material as revealed by SDS PAGE analysis (Fig. 3B). This minor contaminant reacted with anti-54/55 and lay in the acidic "arc" of this subunit's breakdown products as revealed by two-dimensional gel analysis (13). This 55K protein was able to be polymerized into IFs by removal of the 8 M urea by dialysis and incorporation into physiological salt buffers (e.g., 170 mM NaCl as in PBS$_a$).

Immunological Identification of Purified Proteins

In addition to identification of IFAP-300K and 55K on the basis of M_r, immunoblot analysis of SDS gels of these fractions with monoclonal anti-IFAP-300K and anti-54/55 confirmed (a) that the 300K species was, in fact, IFAP-300K, and (b) the absence of 54/55K subunit proteins (or oligomers thereof) in the 300K fraction and vice versa (Fig. 4). Multiple high molecular weight polypeptides in addition to the 300K species were present in this purified fraction. On the basis of their immunoreactivity with anti-IFAP-300K (Fig. 4B), these polypeptides presumably represented derivatives of the originally purified 300K. This was in agreement with the previous finding of multiple immunoreactive bands in native IF prep-

arations and their identical enzymic peptide map patterns (40).

Characterization of IFAP-300K

Determination of the pI value for the intact 300K molecule was best accomplished by chromatofocusing of the enriched Sephacryl S-400 peak fraction (Fig. 5). On the basis of SDS PAGE assessment of the focused fractions, the molecule was eluted with its peak center at an apparent pI of 5.35. This value was comparable to that for 54K and 55K. The technique of chromatofocusing was also sometimes used as an alternative purification method, but the recovery of protein was very inefficient, due to the multiple steps (i.e., ammonium sulfate precipitation, dialysis, lyophilization) necessary to remove the amphoteric carrier molecules used in establishing the pH gradient.

Two-dimensional gel electrophoresis (Fig. 6A) did not identify a 300K species in native IF preparations or purified fractions. However, in combination with immunoblot analysis of the two-dimensional gels, an anti-IFAP-300K-reactive doublet spot at ~200-mol-wt, pI 5.5, was identified (Fig. 6B). Unfocused, immunoreactive material was also present in the second dimension SDS gel at the level of the origin of the first dimension gel in the ~300-mol-wt region. As previously described for one-dimensional SDS PAGE immunoblots using anti-IFAP-300K (Fig. 4), reactive species in the 200,000–300,000-mol-wt range represented proteolytic products of IFAP-300K on the basis of their reaction with anti-IFAP-300K and by their homologous one-dimensional enzymic peptide map patterns (40).

This lack of penetration of the first dimension (isoelectric focusing) gel occurred even when samples (a) had been clarified of large aggregates by centrifugation at 265,000 g for 1 h; (b) were focused on gels of various polyacrylamide concentrations, and (c) were prepared and focused in the presence of non-ionic detergents (Nonidet P-40, Triton X-100), urea, glycerol, ethylene glycol, reducing and chelating agents, or under native conditions.

One-dimensional peptide mapping by the limited proteolysis technique (2) has demonstrated that IFAP-300K has a peptide pattern markedly different from those of the 54K and 55K IF structural subunits (40). However, the total amino acid compositions of purified IFAP-300K and the two IF structural subunits demonstrated extensive similarity (Table I). The preponderance of potentially acidic vis-a-vis basic residues (27 mol% vs. 14 mol%) was consistent with the acidic nature of the polypeptide. The compositional values of column-purified IFAP-300K and 55K compared favorably with
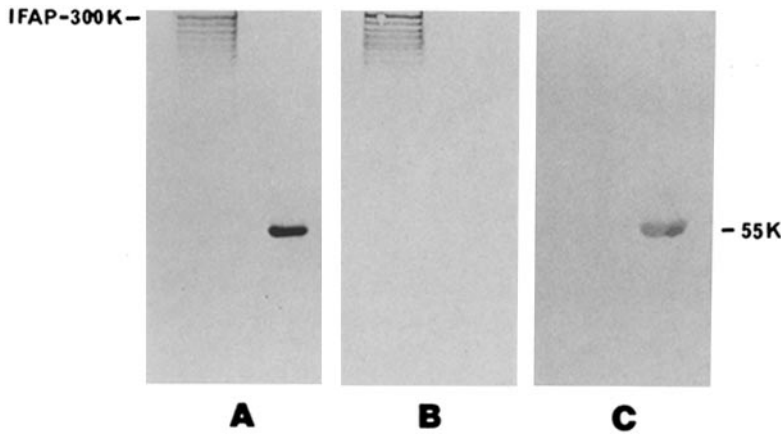
FIGURE 4 Immunoblot analysis of purified IF fractions. (A) Coomassie Blue-stained SDS gel of purified 300K (left lane) and 55K (right lane). (B) Nitrocellulose transfer of A immunostained with anti-IFAP-300K identified the polypeptide as IFAP-300K. The multiple bands seen in A were all immunopositive, suggesting their derivation from IFAP-300K (see text). (C) Nitrocellulose transfer of A immunostained with anti-54/55.
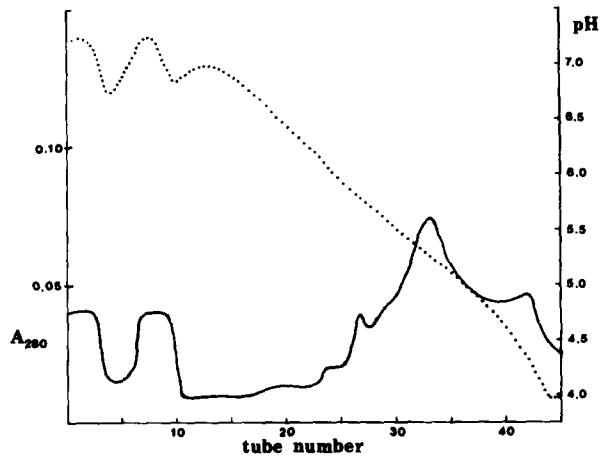

FIGURE 5 Chromatofocusing of IFAP-300K. Chromatofocusing from pH 7.4 → 4.0 of the 300K-enriched gel filtration fraction established an apparent pI of 5.35 for IFAP-300K (tube #33). The dotted line represents the pH gradient values.

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

	IFAP-300K*	300K[‡]	Vimentin (55K)*	Desmin (54K)[‡]
	mol %	mol %	mol %	mol %
Asp	9.81	9.69	12.11	9.75
Thr	5.59	5.76	5.03	4.63
Ser	7.19	6.69	9.04	9.90
Glu	17.08	17.59	18.18	16.13
Pro	6.57	6.82	4.67	5.78
Gly	8.99	8.90	6.53	7.72
Ala	9.87	9.97	8.05	9.02
Val	3.82	3.23	6.07	6.02
Met	1.29	2.17	0.32	0.90
Ile	1.97	1.96	1.82	3.00
Leu	9.11	9.21	9.45	7.75
Tyr	2.43	2.49	2.37	2.24
Phe	2.69	2.50	2.35	2.92
His	1.91	2.23	1.02	0.66
Lys	5.20	3.93	4.04	4.45
Arg	6.48	6.86	8.75	8.95

Values are averages of two 24-h hydrolyses for each polypeptide. Cys and Trp values were not determined.
* Column-purified polypeptides.
[‡] Polypeptides eluted from SDS gel slices. Values from Yang et al. (40).

those previously determined for the respective gel-slice material (40).

Despite the aggregation problems encountered with isoelectric focusing, IFAP-300K remained very "soluble" when dialyzed from urea-containing column buffers into low salt buffers (in particular, 5 mM sodium phosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, the buffer used for IF disassembly into protofilaments [30, 42]). Centrifugation at 130,000 g for 1 h produced minimal pelletable material, a situation confirmed by the continued presence of an essentially pre-centrifugation concentration of IFAP-300K material in the supernatant when analyzed by absorbance at 280 nm, negative staining, and SDS PAGE (see below).

IFAP-300K Morphology

Electron microscopic examination of negatively stained preparations of IFAP-300K in low salt buffer revealed a uniform dispersion of particles, the majority of which were composed of one or two spherical or slightly elliptical units measuring ~16 × 20 nm (Fig. 7A). In PBS$_a$ (NaCl = 170 mM), larger, non-filamentous aggregates predominated (Fig. 7B). The substructure of these aggregates consisted of units identical to the 16 × 20-nm particles seen in low salt preparations. This suggested that under higher salt conditions the individual particles were caused to aggregate to form the larger structures. Immunogold labeling of these preparations dem-

onstrated specific localization by anti-IFAP-300K (Fig. 7C), thereby confirming their composition.

The distinctive, nonfilamentous morphology of purified IFAP-300K vis-a-vis IFs reiterated the non-identity of these two elements. It should be noted that the size and internal structure of IFAP-300K aggregates in PBS$_a$ coincided with that of the amorphous, proteinaceous material localized in situ (40) and in native IF preparations (Fig. 1) by anti-IFAP-300K. This suggested that the in vitro aggregates may represent the functional form of the protein and that the purification process did not irreversibly destroy the morphology or re-association properties of the molecule.

In Vitro Recombination of IFAP-300K with IF

Using purified IFAP-300K and 55K from BHK-21 cells, recombination experiments were undertaken to determine whether these species represented the minimum number of polypeptides required for regenerating a 300K/IF complex and, if so, whether 300K were distributed on IFs in the same manner as seen in situ and in native IFs; and also to see whether 300K had any effect upon the kinetics of IF polymerization in vitro.
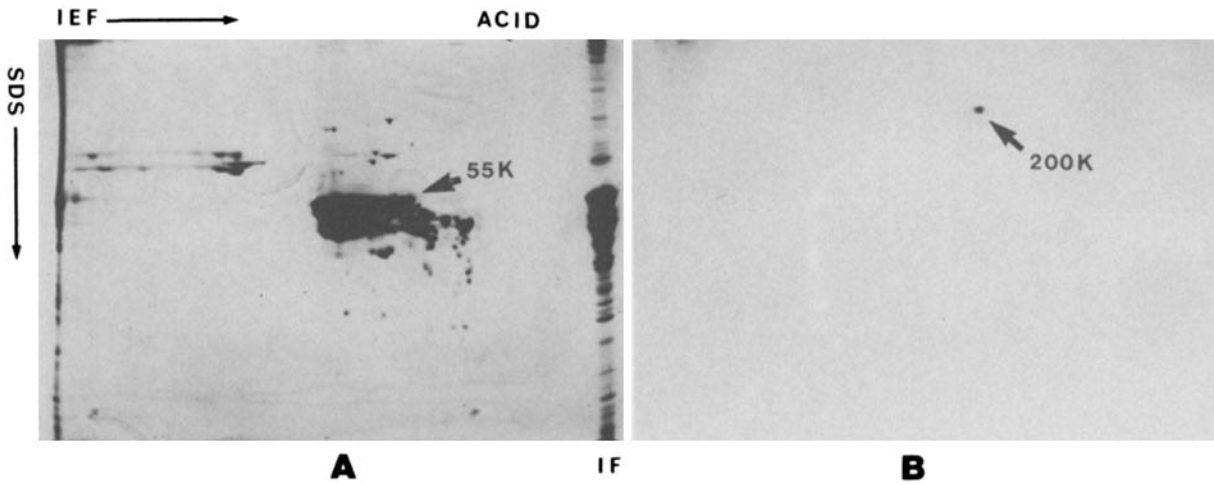
FIGURE 6 Two-dimensionsal gel analysis of IFAP-300K. The charge composition of IFAP-300K was investigated in native IF preparations. (A) Overloaded sample. One-dimensional marker lane of the IF preparation is shown along right side of gel. Coomassie Blue–stained gel. (B) Nitrocellulose transfer of A immunostained with anti-IFAP-300K. A 200K doublet at pI 5.5 was the only reactive species; the 300K species did not enter the first dimension isoelectric focusing gel (note heavy staining at origin). pH range 7.5–4.5. Other pH ranges yielded the same, relative spot positions and pI values.
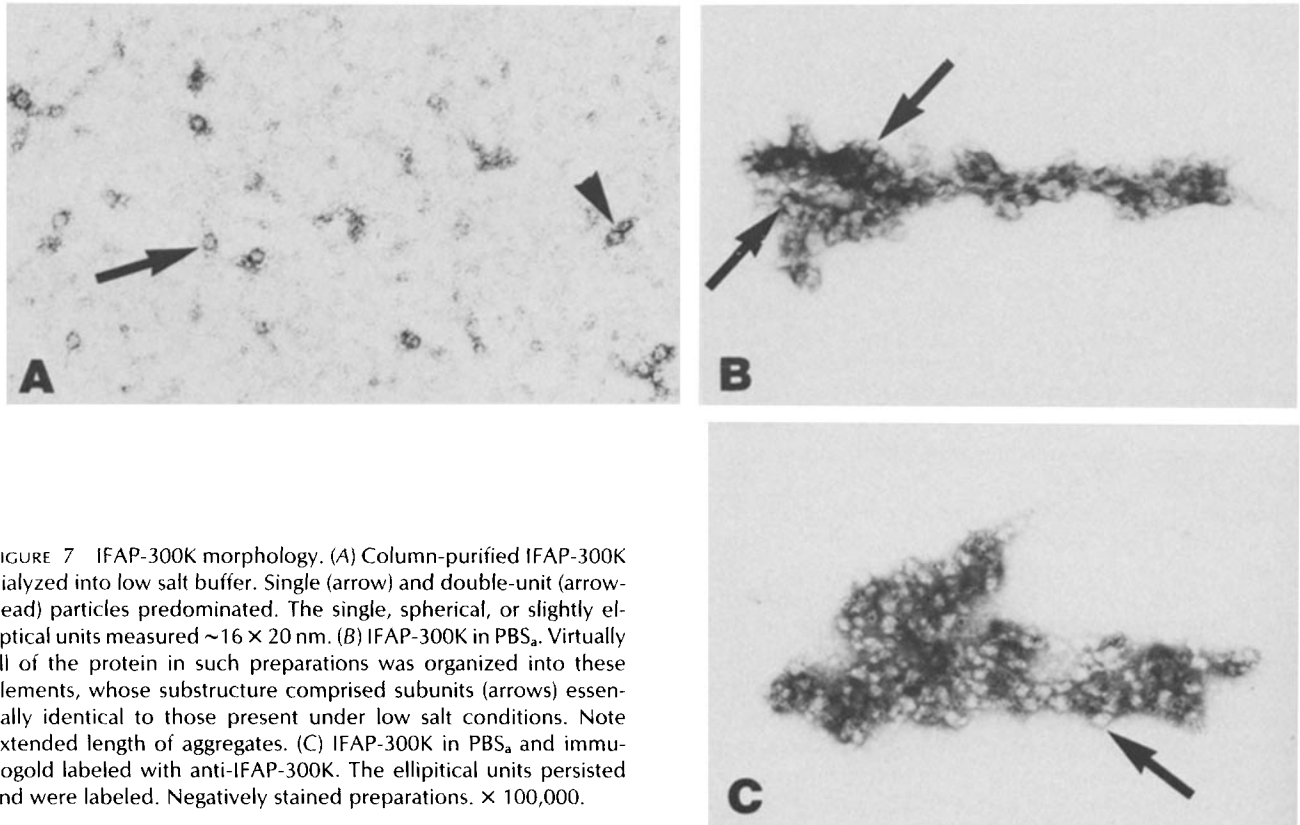

FIGURE 7 IFAP-300K morphology. (A) Column-purified IFAP-300K dialyzed into low salt buffer. Single (arrow) and double-unit (arrowhead) particles predominated. The single, spherical, or slightly elliptical units measured ~16 × 20 nm. (B) IFAP-300K in PBS$_a$. Virtually all of the protein in such preparations was organized into these elements, whose substructure comprised subunits (arrows) essentially identical to those present under low salt conditions. Note extended length of aggregates. (C) IFAP-300K in PBS$_a$ and immunogold labeled with anti-IFAP-300K. The ellipitical units persisted and were labeled. Negatively stained preparations. × 100,000.

The rationale of the experiment addressing the first question was that if 300K was added to polymerizing IFs and became associated with them, it should be sedimented with the IFs by centrifugation. This was indeed the result obtained. As shown in Fig. 8A, lane f, both polypeptides (55K and 300K) were pelleted from the recombination mixture by ultracentrifugation. While 55K was not pelletable from low salt buffers (data not shown), its polymeric state in 170 mM NaCl (i.e., IF) clearly was (Fig. 8A, lane d). However, 300K was not pelletable from 170 mM NaCl buffer (Fig. 8A, lane b). Thus, the finding of this polypeptide in the pellet from the recombina-

tion mixture was not due to insolubility in the NaCl buffer, but rather to some type of interaction with the pelletable IF. This result was also obtained when 300K was added to preformed IF. The weight ratio of 300K to 55K used in the recombination mixture was 0.07. This represented the approximate ratio determined for native IF preparations on the basis of protein band stain intensity. The ratio observed in the co-sedimented pellet was slightly lower (Fig. 8A, lane f). The same results were obtained when IFAP-300K was added to pre-formed, rather than to actively polymerizing, IFs. That co-sedimentation with filamentous proteins was not a non-

-IFAP-300K

-55K

a b c d e f

A

-IFAP-300K

-55K

ACTIN-

a b c d

B
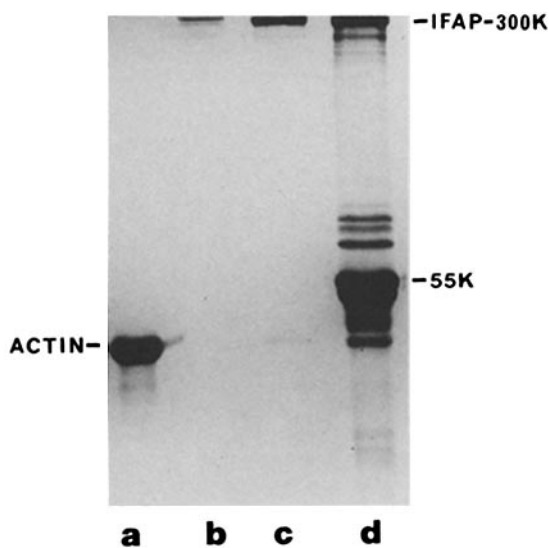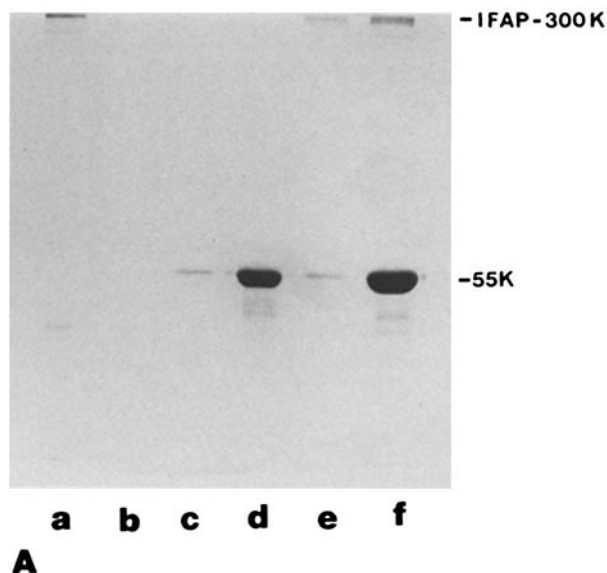
FIGURE 8 Co-sedimentation of recombined IFAP-300K with 55K IF. (A) SDS gels of products of 100,000 g centrifugation of specimens dialyzed into PBS$_a$. IFAP-300K supernatant (a) and pellet (b). The molecule remained soluble. 55K supernatant (c) and pellet (d). Essentially all of the 55K was polymerized into pelletable IFs. Recombined IFAP-300K/55K supernatant (e) and pellet (f). The otherwise soluble IFAP-300K was now pelletable, presumably by virtue of its having become physically associated with the polymerized IF. (B) SDS gels of corresponding experiment in which actin was substituted for 55K. (a) Pellet from IFAP-300K/actin polymerization mixture. Only F-actin was sedimented. (b) Supernatant from IFAP-300K/actin polymerization mixture. (c) Heavier loading of b to demonstrate only a trace amount of actin remaining unpolymerized, while virtually all of the IFAP-300K remained in this fraction. (d) Native IF preparation.

specific property of IFAP-300K was shown by its inability in similar recombination experiments to pellet with F-actin polymerized from mixtures of the molecule and G-actin (Fig. 8 B).

The highly purified state of the 55K subunit protein obtained by the chromatographic procedure described above

permitted the in vitro polymerization of homopolymer IFs. These IFs had sharply defined borders, and the preparations contained minimal background debris as defined by negative stain electron microscopy (Fig. 9 A). When IF were polymerized from 55K in the presence of IFAP-300K, IFs were formed which were identical to those generated from 55K alone, with the exception that clusters of material, apparently corresponding to molecules of IFAP-300K, were clearly visible in areas of close IF–IF association (Fig. 9 B). The same finding was obtained when pre-formed IF, rather than polymerizing IF, were used. This arrangement was identical to that observed for IFAP-300K/IF in situ and in native IF preparations. Moreover, the clarity of these preparations allowed visualization of IFAP-300K/IF configurations which were partially obscured in situ and in native IF. Most obvious were apparent connecting links between slightly separated IF (Fig. 9 C). The lengths of such "bridges" (up to ~50 nm) were comparable to the length of purified IFAP-300K aggregates in the presence of PBS$_a$ (Fig. 7, B and C).

To further clarify the structural contribution of each purified component to the recombined morphology, decoration of the elements by incubation with the specific antibodies was performed. Treatment with anti-54/55 produced a continuous decoration of the IF between crosspoints in an apparent spiral pattern (Fig. 10 A). This was in marked contrast to the complete absence of any IF labeling by monoclonal anti-IFAP-300K, which caused a significant mass increase only at points of interaction between IF (Fig. 10 B). This latter point was confirmed by the appearance of gold particles at these points following subsequent incubation with gold-conjugated secondary antibody (Fig. 10 C). These findings verified the co-sedimentation data described above and the location of IFAP-300K seen both in situ and in vitro.

To determine whether IFAP-300K had any effect upon the kinetics of polymerization of 55K into IFs, polymerization was turbidimetrically monitored in both the presence and absence of 300K. Under the conditions used, the respective optical density curves were indistinguishable in shape, amplitude, and time course, suggesting neither a promoter nor suppressor effect by IFAP-300K.

DISCUSSION

In this study, the 300K protein previously described with respect to its in situ distribution and interaction with BHK cell IFs (40) has been purified from the high salt/detergent-insoluble material obtained from this cell type, and its recombination with purified IFs has been demonstrated in vitro. Indirect immunogold labeling with the monoclonal anti-IFAP-300K characterized in the previous study confirms that IFAP-300K in these recombined preparations occupies the same location on the IF as observed in situ (40) and in native preparations, i.e., at certain points of close association between IFs. Furthermore, the absence of co-sedimentation with F-actin in vitro suggested the IFAP-300K/IF interaction is specific.

Several characteristics of IFAP-300K which complicate purification of the molecule may be reflective of at least one of its possible roles, namely that of an IF–IF linking protein. The lability of this protein increases substantially with each successive step in the purification protocol. This is seen in a tendency toward self-aggregation under certain conditions, as well as in an apparent susceptibility to proteolytic degradation.
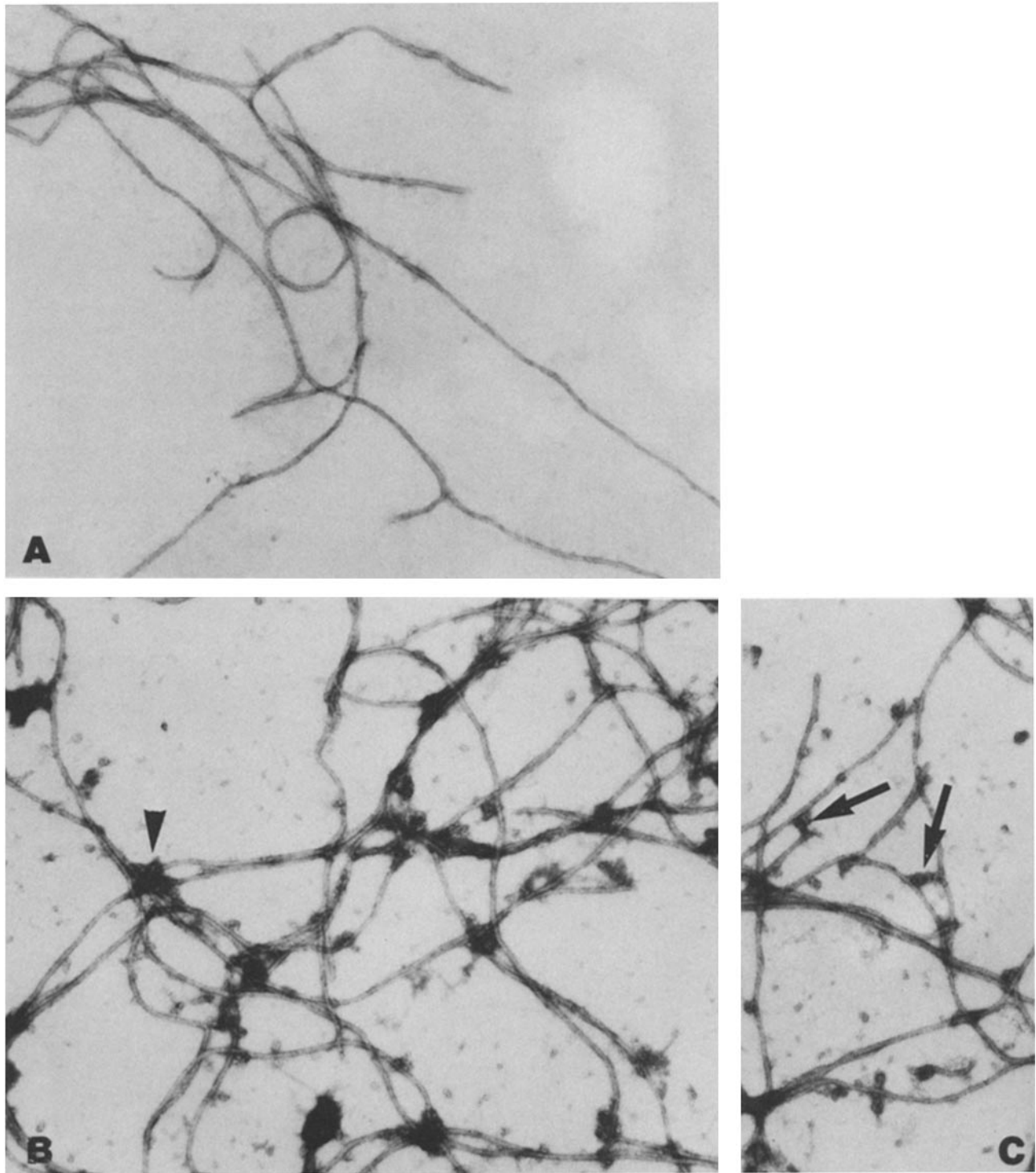
FIGURE 9   Morphology of recombined IFAP-300K and 55K IFs. (A) 55K polymerized into IFs in PBS$_a$. Note sharp borders of IFs and equal density at points of contact between filaments. Represents morphological counterpart of specimen in Fig. 8A, lane d. (B) 55K polymerized into IFs in the presence of IFAP-300K in PBS$_a$. Morphological counterpart of specimen in Fig. 8A, lane f. Compared with A, amorphous material (arrowhead) was now present at a majority of contact points between filaments. This material, apparently corresponding to IFAP-300K, appeared to be composed of subunits identical to those observed in aggregates of purified IFAP-300K (see Fig. 7, B and C). (C) Another view of recombined IFAP-300K/IF showing apparent "bridges" of aggregated subunits between individual IFs. The length of such links was comparable to those of purified IFAP-300K aggregates (see Fig. 7, B and C). Negatively stained preparations. × 67,000.

The IFAP-300K–enriched fraction elutes from the S-400 gel filtration column at a point seemingly higher in molecular weight than predicted, even allowing for the loss of filtration capacity caused by the presence of 7.2 M urea. This is con-

sistent with its minimal penetration of low porosity isoelectric focusing gels and its morphological appearance as large particles in the presence of physiological salt concentrations. Indeed, the localization of IFAP-300K in its apparently native
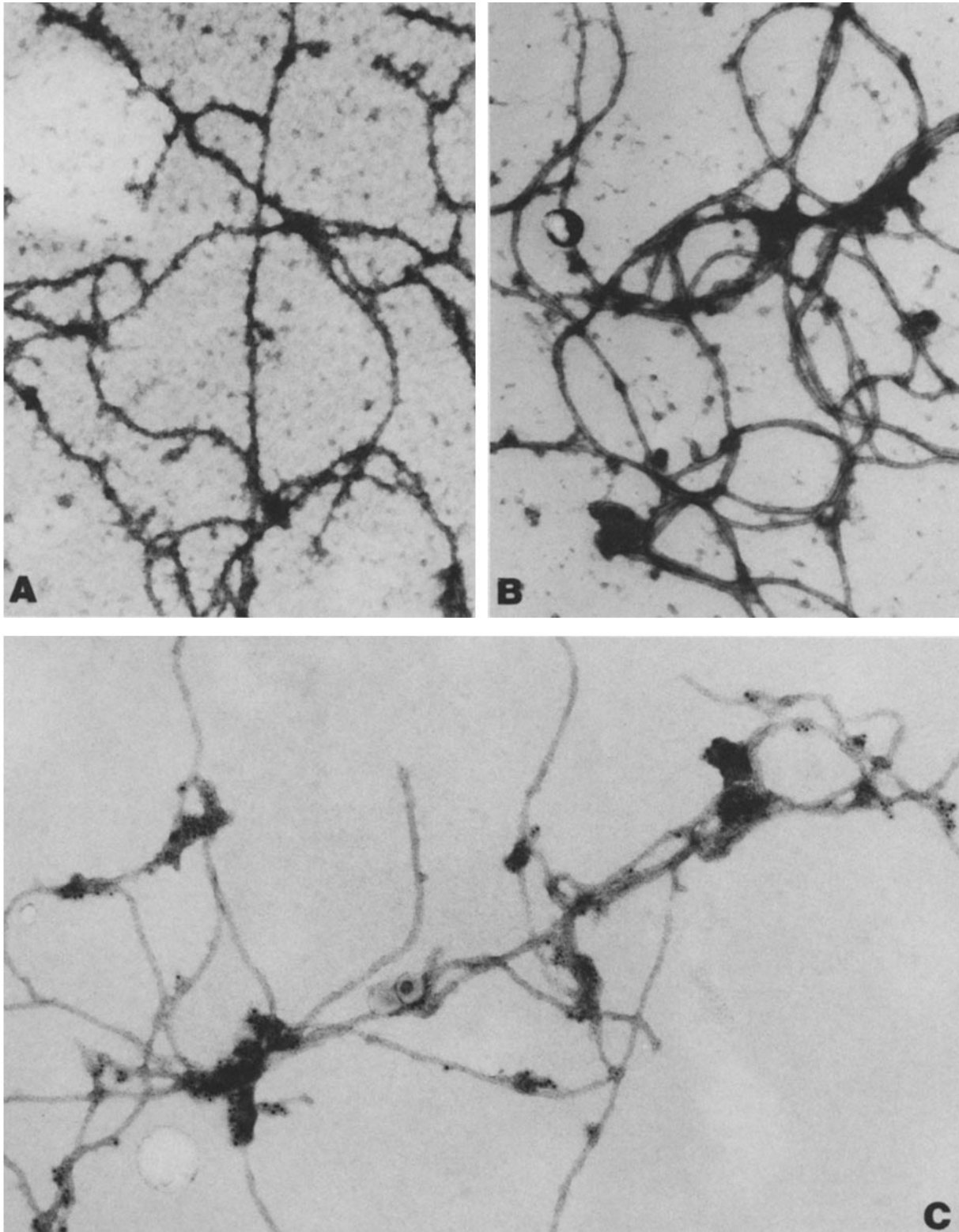
FIGURE 10 Antibody decoration of recombined IFAP-300K and 55K IFs. Recombined specimens as in Fig. 10, B and C, following incubation with: (A) anti-54/55; (B) anti-IFAP-300K; and (C) anti-IFAP-300K followed by gold-conjugated secondary antibody. The filament borders in A were blurred throughout their lengths, apparently due to attached IgG molecules. Note spiral pattern of attachment. Note absence of IF decoration in B. The expected decoration in B of the proteinaceous material between IFs by anti-IFAP-300K was apparent as an increase in the size of these clusters and in their density. These elements were indeed labeled by subsequent immunogold staining, as seen in C. Such localization was identical to that observed in native IF preparations (Fig. 1A) and in situ (40). Negatively stained. (A and B) × 71,000. (C) × 104,000.

state by the immunogold method (i.e., both in situ (40) and in native IF preparations [Fig. 1 *A*]) reveals that it is associated with IFs in the form of large clusters similar to those observed in preparations of purified IFAP-300K under nondenaturing conditions (Fig. 7 *B*). Such intermolecular cohesiveness would be a desirable property for a putative linking element.

With respect to proteolytic degradation in the purified state, the interaction of IFAP-300K with the IF may confer on the associated protein some degree of protection from cellular proteases. This susceptibility appears to be a commonly encountered problem in purifying putative high molecular weight IFAPs, its having been described for synemin (14, 27), paranemin (1), and plectin (37). In many instances, such breakdown may not be detectable in SDS polyacrylamide gels by protein stains but becomes evident by staining with antibody probes. It is, therefore, necessary to routinely include chelators (EDTA, EGTA) and protease inhibitors (phenylmethylsulfonyl fluoride, n-tosyl-L-arginine methyl ester) in all buffers. In this study, the exclusion of disulfide reducing agents (β-mercaptoethanol, DTT, dithioerithreitol) at certain stages appears also to limit proteolysis, perhaps by diminishing the activity of sulhydryl-dependent proteases.

Several investigators have addressed the problem of analysis of these high molecular weight proteins by isoelectric focusing and two-dimensional PAGE (14, 22, 37). Most studies have shown difficulty with penetration of the protein into the isoelectric focusing gel, even though the electrophoretic system contains 9 M urea and a reducing agent, and the gel consists of <4% polyacrylamide (23). For example, synemin has a molecular weight of 230,000 on SDS gels and 980,000 in Tris/KCl buffer (27), but its size in urea is not known. At 9 M urea concentration the IFAP-300K polypeptide chains should theoretically be completely unfolded (3, 4), and, therefore, migration into such a low porosity gel would seem to be possible. Indeed, in more recent studies (27) synemin focused well. One could speculate that for IFAP-300K, other types of protein–protein interaction not controlled by these denaturants may be operative. For example, divalent cation-dependent cross-linkage or hydrophobic interaction could generate aggregates of such size as to prevent penetration into the gel matrix. However, the inclusion here of EDTA to chelate divalent cations and of ethylene glycol and non-ionic detergents to reduce the polar nature of the system does not markedly improve the electropherograms. Nonetheless, it is possible to focus at pI 5.5 an immunologically defined derivative of IFAP-300K on two-dimensional gels. This is a slightly more basic value than that of 5.35 obtained for the intact molecule by chromatofocusing. These findings are consistent with its high proportion of potentially acidic amino acid residues as revealed by total amino acid analysis (Table I). Although IFAP-300K's pI and amino acid composition are remarkably similar to those of 54K and 55K, the evidence overwhelmingly shows that IFAP-300K does not represent an oligomer of 55K. This evidence is (a) the lack of immunologic cross-reactivity using antibodies of two different specificities; (b) the nonhomologous peptide maps (40); (c) the different morphologies; and (d) the different morphological contributions to IF structure. Nonetheless, their highly similar chemical properties may suggest that they are members of a larger family of evolutionarily related proteins.

The fact that IFAP-300K is able to re-associate with purified 55K IFs in recombination experiments to form the same morphological configurations as seen in situ and in vitro

demonstrates the self-assembly properties of this complex in generating an interconnected network. The fact that no differences in morphology or immunolocalization of IFAP-300K are seen when the protein is added to polymerizing or preformed IFs suggests that the molecule is a surface-associated protein and not an integral IF subunit. The apparent joining property of IFAP-300K as it exists in large aggregates associated with IFs contrasts with the proposed non-cross-linking and single-molecule form of association with IFs seen for epinemin (18).

Whether IFAP-300K associates preferentially with preformed IFs or with their protofilamentous state is not known. This becomes significant in determining whether IFAP-300K may dictate the points of interaction between individual IFs by virtue of its binding, or whether points of interaction occur randomly and are subsequently "welded" by IFAP-300K's binding to the crosspoints. It would also be interesting to know whether in vitro modification of the molecule (e.g., phosphorylation, specific proteolytic processing, ligand binding) has any effect upon its interaction with not only vimentin IFs, but also with other classes of IF (e.g., keratin IFs, which are organized into macrofibrils by its naturally associated protein, filaggrin [31]). IFAP-300K's selective association with IFs serves to differentiate this associated protein from the 300K protein plectin for which evidence as to its widespread cell type and organelle association continues to accumulate (39).

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