Different Polypeptides Form the Intermediate Filaments in Bovine Hoof and Esophageal Epithelium and in Aortic Endothelium

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ABSTRACT Polypeptides that form 10-nm filaments in vitro were isolated from three different bovine tissues: the viable portion of the hoof epithelium, the epithelium of the esophagus, and cultured endothelial cells derived from aorta. The seven polypeptides from hoof, the two from esophagus, and the one from endothelial cells were different with respect to mobility in SDS polyacrylamide gels and/or limited proteolytic digestion. Peptide maps of the different filament-forming polypeptides (FFP's) showed that none of the smaller FFP's was a fragment of any of the larger FFP's. Several isomobile fragments were found in the peptide maps of different FFP's,suggesting that they might contain regions of amino acid sequence homology. We present a hypothesis that suggests how the different 10-nm filament-forming proteins may be related.

Intermediate filaments are intracellular filaments present in most, if not all, mammalian cells. They measure 6–10 nm in diameter and are especially prominent as tonofilaments in cells of stratified squamous epithelia, as nuclear cap filaments in a variety of cultivated cells, and as neurofilaments in nerve axons.

In addition to their similar morphology, intermediate filaments from different cell types share many properties, such as x-ray diffraction pattern (8, 26), content of α-helix (18, 26), and amino acid composition (11, 23). Immunofluorescence microscopy, using antibodies to individual intermediate filament proteins, distinguishes four classes of intermediate filaments and suggests that the filaments in different epithelia are related to to each other but not to the filaments in nonepithelial tissues (1, 9, 10, 27). These methods are useful for comparing intact filaments in different tissues. They do not indicate whether individual filament-forming polypeptides (FFP's) have significantly large regions of related amino acid sequence.

Peptide mapping is often the first step in establishing the presence of sequence homology in different polypeptides. Data derived from this method indicate that the keratins within a given epithelial tissue may be related (11, 16), that vimentin and desmin may be related (12), but that the major FFP in neural tissue is different from that in muscle (7).

In the experiments described below, we used one-dimensional peptide mapping to compare FFP's from different tissues. The published immunofluorescence microscopy data suggests that filaments in hoof and esophagus are composed of related FFP's (10, 27) but that two isomobile FFP's from eosphageal and endothelial cell filaments are unrelated (1, 9, 10). The principal objectives of our studies were to determine whether (*a*) FFP's of the same molecular weight but from different tissues were the same, (*b*) any of the smaller FFP's were fragments of the larger FFP's, and (*c*) different FFP's yielded peptide maps that were consistent with regions of amino acid sequence homology.

MATERIALS AND METHODS

Tissue Preparation and Protein Extraction

Hoof, esophagus, and aorta were obtained from freshly slaughtered calves. Hoof epithelium was dissected into three layers: the outer glassy layer, consisting entirely of stratum corneum; a 1–2-mm middle layer (the upper viable layer), consisting of cells of the granular layer and upper stratum spinosum; and an inner, pigmented layer, consisting of cells of the basal layer, lower stratum spinosum, melanocytes, and tips of the dermal papillae. Only the upper viable layer was used for these studies because it was the only region of the epithelium that contained significant amounts of each of the hoof FFP's (19). Upper viable hoof epidermis was homogenized for 1 min in a Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) in 2 mM Tris, pH 8.0, with 2 mM ATP, 0.5 mM β-mercaptoethanol (β-ME), and 0.2 mM $CaCl_2$, and centrifuged at 20,000 *g* for 10 min. The resulting pellet was stirred for 2 h at 37°C in 0.05 M Tris, pH 9.0, with 8 M urea and 0.025 M β-ME and centrifuged at 20,000 *g* for 10 min. This urea-soluble extract contained the hoof FFP's.

Esophageal epithelium, separated from underlying connective tissue by heating to 56°C for 30 s, was homogenized for 1 min in a ground-glass homogenizer in 0.05 M Tris, pH 7.4, and centrifuged at 20,000 *g* for 10 min. The pellet was stirred for 2 h at 37°C in 0.05 M Tris-HCl, pH 7.4, with 8 M urea and 0.025 M β-ME and centrifuged at 20,000 *g* for 10 min. This urea-soluble extract contained the esophageal FFP's (20).

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Endothelial cells were obtained from aorta, as described by Gospodarowicz et al. (13), by gently stroking the endothelial surface with a cotton swab and then rinsing the swab with culture medium consisting of Dulbecco's Minimal Essential Medium (Grand Island Biological Company, Grand Island, N. Y.) with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells having the morphology of endothelial cells in culture were grown at 37°C in a humidified atmosphere of 5% CO_2:95% air. Intact nuclear cap filaments were harvested from 10^8 spreading cells by the method of Starger et al. (23) and solubilized in 0.01 M Tris, pH 7.4.

All buffers contained 0.1 mM phenylmethylsulfonyl fluoride. Stock solutions of 10 M urea were deionized before use by passage over a mixed ion exchange resin (Crystalab, Inc., Hartford, Conn.).

SDS Polyacrylamide Gel Electrophoresis

Discontinuous SDS polyacrylamide slab gel electrophoresis was performed on 14 × 14 × 0.15-cm slabs as described by Laemmli (17) with the following modifications: the acrylamide:bisacrylamide ratio in the 12.5% separating gel was 120:1, the acrylamide:bisacrylamide ratio in the 5% stacking gel was 30:0.8. Electrophoresis was performed at room temperature for 6 h at 100 V. Before application to the gels, samples were boiled for 3 min in 0.08 M Tris, pH 6.8, containg 2% SDS, 1% β-mercaptoethanol, and 10% glycerol. Gels were stained with 0.2% Coomassie Blue R-250 in 10% acetic acid-50% methanol and destained in 10% acetic acid-10% methanol.

Isolation of Individual Polypeptides

Single-well, SDS polyacrylamide slab gels, prepared as above, were loaded with extracts containing 100–300 μg of protein. After electrophoresis, gels were stained for 15 min with Coomassie Blue, and strips of gel containing a single protein band were carefully cut from the slab and inserted into tubes fitted with a dialysis bag at one end. Protein was electrophoretically eluted overnight at 2.5 mA/tube and then precipitated with 20% trichloroacetic acid at 4°C and washed with ether.

Limited Proteolytic Digestion of Individual Polypeptides

Limited proteolytic digestion of individual protein bands with either *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) or chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.) was performed as described by Cleveland et al. (4). Each tube containing 30 μg of protein and 1–5 μg of enzyme in a total volume of 50 μl was incubated for 30 min at 37°C. Control tubes, containing enzyme alone, were incubated and analyzed in parallel with the samples. Products of proteolysis were examined on gels as described above except that the separating gel contained 15% acrylamide with an acrylamide:bisacrylamide ratio of 30:0:8. Gels were run for 9 h at 100 V.

Protein Quantitation and Negative Staining and Electron Microscopy of Filaments

Protein was assayed by the method of Bramhall (3) with bovine serum albumin (Miles Laboratories, Inc.) as standard. Suspensions of filaments were spread on carbon-coated mica chips, transferred to carbon-coated holey grids, and stained with 1% uranyl acetate. Specimens were examined with a Siemens 101 electron microscope at 80 keV.

RESULTS

Polypeptide Identification

The major polypeptides that form intermediate filaments in three calf tissues are shown in Fig. 1. Seven polypeptides in the urea extract of hoof have molecular weights ranging from 49,000 to 65,000 and together comprise 60% of total extractable hoof protein. We have designated these polypeptides, from higher to lower molecular weight, K_{1a}, K_{1b}, K_2, K_3, K_4, K_5, and K_6, in keeping with Steinert and Idler's nomenclature (24). They separated six hoof FFP's by polyacrylamide gel electrophoresis and called them keratins K_1–K_6; they found that K_1 had two components (K_{1a} and K_{1b}) which were separable by ion exchange chromatography. Our polyacrylamide gel system separates all seven hoof FFP's. The two polypeptides in the urea extract of esophageal epithelium have molecular weights of 56,000 and 46,000, designated E_1 and E_2, respectively, and together comprise 35% of total extractable protein in esophageal epithelium (20). The major polypeptide in intact, nuclear cap filaments obtained from cultivated endothelial cells has a molecular weight of 56,000 and is ~1% of total extractable protein in these cells. The endothelial cell nuclear cap filaments can be dissolved in 10 mM Tris, pH 7.4.

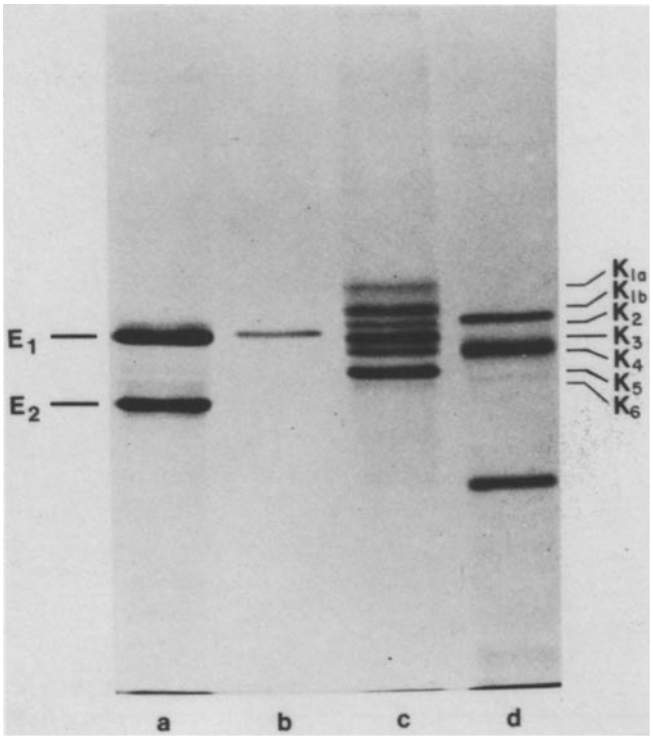

FIGURE 1 12.5% acrylamide SDS gel of: *a*, esophagus, 0.05 M Tris-8 M urea-β-ME extract; *b*, endothelial cell nuclear cap filament preparation; *c*, hoof, 0.05 M Tris-8M urea-β-ME extract; *d*, molecular weight markers: pyruvate kiase (57,000), glutamic dehydrogenase (53,000), glyceraldehyde 3-phosphate dehydrogenase (36,000).

Filament Formation

Filaments measuring 6–10 nm in diameter are formed under appropriate conditions in each of the polypeptide solutions described above. Supernate fractions from high-speed centrifugation (200,000 *g* for 1 h) of solubilized endothelial filaments and of urea extracts from hoof or esophagus contain no filaments. Filaments form in these supernate fractions when urea is removed from the hoof or esophagus extracts by dialysis (Fig. 2) or when the concentration of KCl in the endothelial extract is raised >0.2 M. Filaments formed in vitro are sedimented by high-speed centrifugation, and the pellets contain the same polypeptides in the same relative amounts as in the original extracts. 80–90% of the protein in the original high-speed supernate is recovered in the pellet of hoof or esophageal filaments; 40–50% of the original protein is recovered in the pellet of reassembled endothelial filaments. Evidence presented elsewhere (20, 25) shows that each of the hoof and esophageal polypeptides mentioned above has the capacity to participate in filament formation.

Peptide Mapping of FFP's

Individual FFP's were isolated by preparative SDS polyacrylamide gel electrophoresis and then digested with either

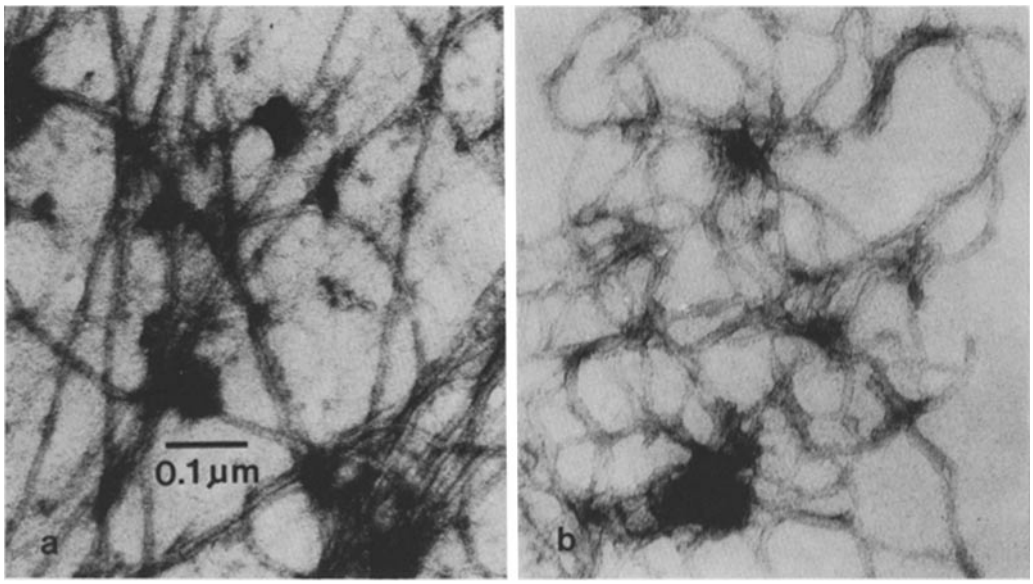

FIGURE 2 Filaments isolated (*a*) as intact nuclear cap filaments from cultivated aortic endothelial cells or (*b*) from a urea-β-ME extract of hoof epithelium. The urea extract (0.5 mg/ml) was dialyzed against 1,000 vol of 0.01 M Tris, pH 7.4, with 0.025 M-β ME for 24 h at 20° C. Suspended filaments were spread on a holey grid and stained with uranyl acetate. × 108,000.

chymotrypsin or *S. aureus* V8 protease. Fragments produced by proteolytic cleavage of the 56,000-dalton polypeptide from endothelial cells, the 56,000-dalton polypeptide from esophageal epithelium, and the 57,000-dalton polypeptide from hoof were separated on SDS polyacrylamide slab gels to determine whether these FFP's were identical (Fig. 3). These three polypeptides yielded distinct cleavage patterns with *S. aureus* protease (Fig. 3) or chymotrypsin (not shown) and, thus, are not identical.

To determine whether the lower molecular weight filament-forming polypeptides in esophagus and hoof were related to the higher molecular weight filament-forming polypeptides in these tissues, we compared the cleavage patterns produced by limited proteolytic digestion of both of the esophageal proteins and five of the hoof keratins. Digestion of the lower molecular weight esophageal polypeptide with *S. aureus* protease (Fig. 4) or chymotrypsin (not shown) produces many fragments which do not have counterparts of identical molecular weight in the digests of the higher molecular weight esophageal polypeptide. Likewise, digestion of each hoof polypeptide with either *S. aureus* protease (Fig. 5) or chymotrypsin (not shown) produces many fragments which do not have counterparts of identical molecular weight in the digests of any of the other hoof polypeptides. It appears, therefore, that each of these hoof and esophageal polypeptides is distinct, and it is unlikely that any one is derived from any of the others. As indicated by the marks in the margins on Fig. 3 and Fig 5, there are some similarities in the cleavage patterns of many of these polypeptides. The possible significance of these similarities is discussed below.

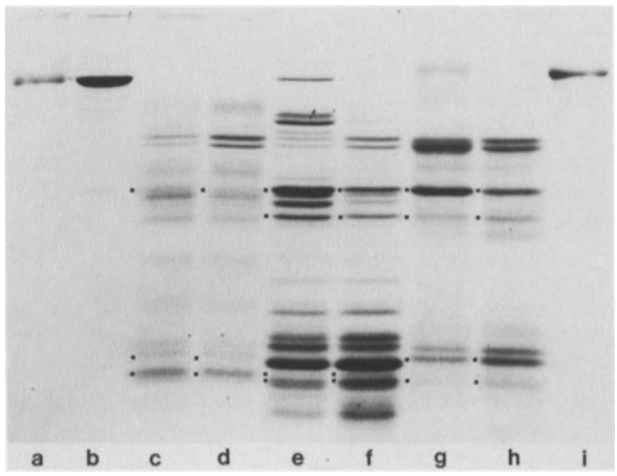

FIGURE 3 15% acrylamide SDS gel comparing proteolytic digests of polypeptides of similar molecular weight. *a*, Purified E_1 (5 μg); *b*, crude nuclear cap filament preparation (20 μg); *c*, E_1 with 1 μg of *S. aureus* protease (SAP); *d*, E_1 with SAP (5 μg); *e*, nuclear cap filament polypeptide with SAP (1 μg); *f*, nuclear cap filament polypeptide with SAP (5 μg); *g*, K_2 with SAP (1 μg); *h*, K_2 with SAP (5 μg); *i*, purified K_2 (5 μg). Dots to the left of a lane indicate fragments present in the proteolytic digests of at least two different polypeptides. Digestion of the crude preparation in lane *b* yields a pattern of fragments identical to that from the digestion of gel-purified nuclear cap filament protein, as shown in lanes *e* and *f*.

DISCUSSION

We have isolated filament-forming polypeptides from three bovine tissues: hoof, esophagus, and aorta. Cells in the stratified squamous epithelium of calf hoof are of ectodermal origin and contain abundant intracellular tonofilaments. Seven FFP's have been identified in calf hoof (24); each can participate in the in vitro assembly of intermediate filaments (25). Cells in the stratified squamous epithelium of calf esophagus are of endodermal origin and contain skeins of intracellular tonofilaments. Two FFP's have been identified in esophageal epithelium; like the hoof FFP's, both of the esophageal FFP's participate in the in vitro assembly of intermediate filaments (20). Aortic endothelial cells are of mesodermal origin, are easily cultivated (13), and have a prominent, perinuclear accumulation of intermediate filaments (2). With the method of Starger et al. (23), we isolated intact intermediate filaments from cultivated endothelial cells and identified a single FFP in these filaments.

We used one-dimensional peptide mapping to compare frag-

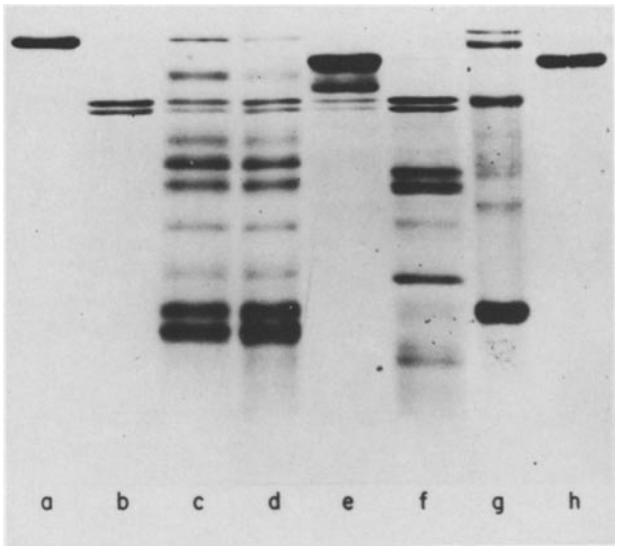

FIGURE 4 15% acrylamide SDS gel comparing proteolytic digests of esophageal polypeptides. *a*, Purified E_1 (10 μg); *b*, SAP (5 μg) incubated alone; *c*, E_1 with SAP (2 μg); *d*, E_1 with SAP (4 μg); *e*, E_2 with SAP (1 μg); *f*, E_2 with SAP (5 μg); *g*, molecular weight markers as in Fig. 1 plus lysozyme (14,000); *h*, purified E_2 (10 μg).

ments produced by limited proteolytic digestion of each FFP. Our rules for interpreting the data were that: (*a*) polypeptides were the same if the fragment maps were the same, (*b*) a precursor-product relationship existed if all the fragments of one polypeptide were a subset of the fragments of another, (*c*) polypeptides were unrelated if none of the fragments of one were a subset of the other, and (*d*) different polypeptides might have regions of sequence homology if they yielded more than one or two isomobile fragments. This method would overestimate similarities because fragments are compared only with regard to size. In contrast, the method can compare only fragments >60–80 amino acid residues in length and, therefore, might miss smaller similarities.

With these criteria and limitations in mind, the one-dimensional peptide maps in Fig. 3 reveal unique digestion products that distinguish three 56,000–57,000-dalton FFP's from each other. There are several shared fragments of identical mobility that could represent large segments of sequence homology. The same is true for comparative maps of the hoof FFP's (Fig. 5) and the esophageal FFP's (Fig. 4) which show that each is distinct, that no one is a precursor or product of any of the others, but that there are isomobile fragments in the digests which could represent regions of sequence homology.

Data in the literature point toward the existence of regions of homologous sequence in different FFP's. There are similarities in the limited proteolytic digests of human epidermal FFP's (11) and in vimentin and desmin-type FFP's in different tissues (12). Precipitating antibodies to individual human (11) or rat (14) epidermal FFP's cross-react with other FFP's from the same tissue. FFP's from different tissues also have similar amino acid composition (11, 23).

It is evident from these data that there are many different polypeptides that can form intermediate filaments. These polypeptides share many properties and, on the basis of peptide mapping data, may contain regions of amino acid sequence homology. The filaments formed by these polypeptides share many properties but can be distinguished on the basis of others.

Comparison of intermediate filaments formed from different FFP's has demonstrated many similarities as well as some important differences in these filaments. All filaments so far examined have similar morphology (26), similar proportions of protein in an α-helical configuration (18, 26), and all yield an α-keratin-type x-ray diffraction pattern (8, 26). This diffraction pattern indicates that the polypeptides in each of these filaments have significant amounts of α-helix in a coiled-coil configuration (6).

Different intermediate filaments have different solubility properties. Vimentin-type and nuclear cap intermediate filaments are dissociated in low ionic strength buffers (9, 23); epithelial (10, 20, 25), desmin (5, 12, 22), and neurofilaments (15, 21) are only dissociated by SDS or high concentrations of urea. Immunofluorescence microscopy studies, in which an antibody to FFP's is bound to filaments *in situ*, suggest that there are four antigenically distinct classes of intermediate filaments: those found in epithelial cells, those in muscle cells, those in nonmuscle mesenchymal cells, and those in neuronal cells (1, 9).

Is there a way to reconcile the data that indicate similarities in intermediate filaments and FFP's with the data that indicate differences in intermediate filaments and FFP's?

If different FFP's were to have regions of amino acid sequence homology, these homologous sequences might explain the similarities in the filaments formed by these FFP's. In the absence of any comparative sequence data, we propose a hypothesis which is capable of accommodating the available data and which could be helpful in directing future comparisons of different FFP's and the filaments which they form.

We hypothesize that different FFP's are organized in a manner analogous to immunoglobulin molecules, in that they have "constant" regions of amino acid sequence homology and "variable" regions of differing amino acid sequence. The constant region(s) of different FFP's would determine the properties that are shared by the filaments they form. The variable regions would determine differences in the properties of the filaments they form. One property shared by all intermediate filaments so far examined is their x-ray diffraction pattern. The hypothesis predicts that the constant region(s) would be in the α-helical portions of the molecule, because that is the region responsible for the diffraction pattern. The remaining nonhelical portions of these molecules would have variable sequences

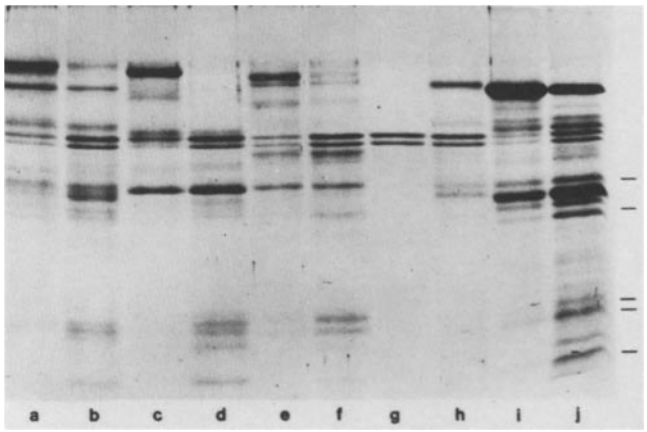

FIGURE 5 15% acylamide SDS gel comparing proteolytic digests of hoof polypeptides. *a*, K_{1a} with SAP (1 μg); *b*, K_{1a} with SAP (5 μg); *c*, K_{1b} with SAP (1 μg); *d*, K_{1b} with SAP (5 μg); *e*, K_3 with SAP (1 μg); *f*, K_3 with SAP (5 μg); *g*, SAP (5 μg) incubated alone; *h*, K_4 with SAP (5 μg); *i*, K_5 with SAP (1 μg); *j*, K_5 with SAP (5 μg).

that would make the filaments they form distinguishable with regard to solubility, immunochemical reactivity, and possibly function.

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