

Isolation and Characterization of Keratin-like Proteins from Cultured Cells with Fibroblastic Morphology

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ABSTRACT Intermediate filaments (IF) isolated from a variety of cultured cells, conventionally described as fibroblasts, are composed predominately of proteins of molecular weights of 54,000 and/or 55,000. Less than 15% of the protein found in native IF preparations from these cells is composed of three to four polypeptides of molecular weights 60,000–70,000. We have investigated some biochemical and immunological properties of these proteins isolated from BHK-21 and mouse 3T3 cells. They are capable of forming paracrystals that exhibit a light/dark banding pattern when negatively stained with uranyl acetate. The dark bands are composed of longitudinally aligned ~2-nm-diam filaments. The center-to-center spacing between either dark or light bands is 37–40 nm. These dimensions are consistent with the secondary structure of IF polypeptides and suggest that the dark bands represent lateral alignment of α -helical coiled-coil domains. Immunoblotting, secondary structure, as well as amino acid composition data indicate that the 60,000–70,000-mol-wt paracrystal polypeptides are similar to keratin. Thus, polypeptides with biochemical and immunological properties of epidermal keratin are present in cells normally considered to be fibroblasts.

Intermediate filaments (IF)¹ represent a family of heterogeneous α -type proteins within the k-m-e-f class which form a major portion of the cytoskeleton of many types of cells (see reference 32 for a review). In a few cases in which the amino acid sequence has been determined, significant sequence homologies have been reported (5, 7, 23). However, IF subunit proteins also exhibit significant differences in molecular weight and antigenic properties. Because of this, it has been suggested that cell and tissue types may be subdivided into different classes based on their IF protein composition (11, 13). One major subclass consists of the keratin subunits that are thought to be excluded from nonepithelial cell types (e.g., fibroblasts), but are present in all epithelial cell types (11, 13, 32). Fibroblasts, on the other hand, appear to contain primarily another type of IF protein subunit (termed vimentin or decamin, references 11, 18, 32). The apparently specific immunocytochemical localization of keratin only in epithelial

cells (3, 25, 27) has led some workers to propose that keratin is an epithelial cell marker, and may be used as such in certain types of clinical diagnosis (13).

There is an inherent danger in drawing conclusions primarily from positive or negative immunofluorescence results. Such data are limited by the uncertainties regarding the fate and availability of antigenic determinants in fixed-processed cells. Therefore, a negative immunofluorescence result could be interpreted in many ways, including the possibility that certain fixation-permeabilization protocols destroy some antigenic sites and not others, and the possibility that the three-dimensional conformation of proteins renders certain antigenic sites unavailable for antibody binding in fixed cells. It is therefore necessary to determine, using independent criteria, whether or not cells commonly classified as fibroblasts contain keratin-like proteins. We have addressed this problem using two extensively studied cultured cell lines, baby hamster kidney (BHK-21) and mouse 3T3 cells. These lines were chosen because they are classified as fibroblasts primarily because of their morphology and they have been shown by immunofluorescence criteria to be "keratin-negative" (2). In this report we describe polypeptides obtained from BHK-21

¹ *Abbreviations used in this paper:* BHK-21, baby hamster kidney; β ME, β -mercaptoethanol; IF, intermediate filaments; IFAPs, IF associated proteins; PBSa, Dulbecco's PBS without Ca⁺⁺ or Mg⁺⁺; PMSF, phenylmethylsulfonyl fluoride.

and 3T3 cells that are associated with the major IF subunit fractions. These proteins can be isolated from preparations of native IF by taking advantage of their relative insolubility under conditions in which the major IF subunit polypeptides are solubilized. Under these conditions, these polypeptides frequently form paracrystals that possess a structure consistent with the secondary structure of IF subunit proteins in general. In addition, α -helix determinations, amino acid composition data, and immunochemical data all suggest that these proteins are of the keratin type.

MATERIALS AND METHODS

Preparation of Native IF: Baby hamster kidney (BHK-21/C13) and mouse 3T3 cells were grown in roller bottles as described elsewhere (1, 18). IF were isolated by a modification of the procedure of Starger et al. (18; see also reference 31). Confluent bottles were rinsed with three 15-ml changes of Dulbecco's PBS without Ca^{++} or Mg^{++} (PBSa; 6 mM Na^+/K^+ phosphate, pH 7.4, 0.171 M NaCl, 3.0 mM KCl). Lysing solution (10 ml per bottle) was added and the cell remains were detached within 3 min by rolling the bottles at room temperature. The lysing solution consisted of 0.6 M KCl, 1% Triton X-100, 10 mM MgCl_2 , 1% β -mercaptoethanol (β ME), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mg/ml *p*-tosyl-L-arginine methylester (TAME) in PBSa. The cell remains in the above solution were further dispersed by vigorous stirring. DNase I (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.5 mg/ml and the suspension was incubated on ice for 3–5 min. The suspension was then centrifuged at 1,600 *g* (for 10 min at 4°C). The resulting IF pellet was washed three times by centrifugation for 3 min at 1,600 *g* (4°C) in PBSa containing 0.1 mM PMSF and 1% β ME.

In Vitro Assembly of IF: IF reassembly cycles were performed as follows: The pellets obtained after lysis and three PBSa washes (see above) were washed twice with buffer consisting of 5 mM Tris-HCl, 0.1 mM EDTA, 1.0% β ME, 0.1 mM PMSF, pH 7.6, and resuspended in this buffer (final protein concentration, 200–400 $\mu\text{g}/\text{ml}$). These solutions, containing disassembled IF in the form of protofilaments (31), were centrifuged at 55,000 rpm (250,000 *g*) for 1 h at 15°C (Beckman type 65 rotor for this and all subsequent centrifugations, Beckman Instruments Inc., Palo Alto, CA). The supernatants were made 0.1 M Na^+ phosphate by addition from a 1.0 M stock solution (pH 7.2). IF assembly was allowed to proceed for 1 h at 21°C, and the resulting IF were harvested by centrifugation at 45,000 rpm (150,000 *g*) for 30 min at 21°C.

Paracrystal Formation: For assembly of paracrystals, the resulting pellet of IF (see preparation of native IF above) was washed twice by low speed centrifugation as described above with 20 mM Na^+ phosphate, 1.0% β ME, 0.1 mM PMSF, pH 7.2. The pellet was resuspended by gentle homogenization in a solution consisting of 8.0 M urea (Schwartz/Mann ultrapure Becton Dickinson, Orangeburg, NY), 20 mM Na^+ phosphate (pH 7.2), 1.0% β ME, 0.1 mM PMSF, to a final protein concentration of 300–400 $\mu\text{g}/\text{ml}$. Urea stock solutions were deionized to remove cyanate by stirring for 30 min with a 2% suspension of Bio-Rad AG 501X8(D) ion exchange beads (Bio-Rad Laboratories, Richmond, CA) immediately before use. The solubilized pellet was centrifuged at 55,000 rpm for 30 min at 21°C (Beckman type 65 rotor). The supernatant was equilibrated with buffer consisting of 2 mM Na^+ phosphate, 0.01 mM PMSF, 0.1% β ME, pH 7.2, by rapid gel filtration on prepacked G-25 Sephadex columns (Pharmacia Inc., Piscataway, NJ; PD-10). The resulting 3T3 IF protein solution was immediately adjusted to a final Na^+ phosphate concentration of 15 mM (pH 7.2) and MgSO_4 was added to 0.5 mM using 100 \times stock solutions. For BHK-21 IF solutions, the final Na^+ phosphate (pH 7.2) and MgSO_4 concentrations were adjusted to 12 and 0.2 mM, respectively. The solutions were incubated overnight (8–20 h) at room temperature (21°C) to assemble paracrystals, which were then harvested by centrifugation at 10,000 *g* for 30 min at 21°C. For further purification of paracrystals, a second cycle of disassembly in 8 M urea and reassembly was performed by repeating the above procedure.

Biochemical and Analytical Methods: SDS-PAGE was performed on 7.5% acrylamide slab gels with 4.5% stacking gels according to the procedure of Laemmli (10). For experiments in which protein was recovered from gels for amino acid analysis, bands were visualized by immersion of the gel in 4 M sodium acetate. Protein from the excised gel bands was recovered by electrophoretic elution into dialysis bags attached to gel tubes that were plugged at the bottom with the Laemmli (10) stacking gel preparation. SDS was removed from these solutions by the method of Henderson et al. (8), and amino acid analysis was performed as described by Steinert et al. (21) using a Beckman amino acid analyzer. Glycine was removed from gel samples before amino acid analysis by the method of Steinert (16). Briefly, the procedure involved 4 \times dialysis of the sample vs. deionized water, followed by gel filtration

on G-25 Sephadex in 5% formic acid and lyophilization prior to acid hydrolysis and amino acid analysis. Control experiments have shown that free glycine is completely removed (reference 16, and P. Steinert, unpublished experiments). Percentage α -helix determinations were performed by circular dichroism and optical rotary dispersion measurements as described previously (24).

Immunological Methods: Two 3-kg New Zealand rabbits were injected subcutaneously in the back with 0.6 mg of either mouse epidermal keratin subunit K1 (20) or BHK-21 55,000-mol-wt protein (18) prepared by preparative gel electrophoresis, and emulsified with an equal volume of Freund's complete adjuvant. The rabbits were boosted intramuscularly at 30 d after the initial injection with 0.5 mg of antigen emulsified with an equal volume of Freund's incomplete adjuvant. The rabbits were bled 1 wk after the booster by ear puncture. Serum was obtained by two cycles of centrifugation at 1,900 *g* for 20 min (4°C). Preimmune serum was collected before the initial injection of antigen.

Ouchterlony double immunodiffusion analyses were performed for both antisera and it was determined that the rabbits produced specific antibodies to the mouse epidermal keratin subunit K1 and the BHK-21 55,000-mol-wt protein (14). Preabsorption of the K1 antiserum with 100 $\mu\text{g}/\text{ml}$ of purified K1 resulted in the loss of tonofilament staining in mouse epidermal cells as determined by indirect immunofluorescence microscopy (9). Preabsorption of the BHK-21 55,000-mol-wt antiserum with 100 $\mu\text{g}/\text{ml}$ of BHK-21 IF protein also resulted in the loss of IF staining in BHK-21 cells as determined by indirect immunofluorescence microscopy (data not shown).

IF proteins run on 7.5% slab gels with 4.5% stacking gels (10) were transferred to sheets of nitrocellulose (26). Transferred proteins were visualized as discrete bands on the nitrocellulose paper after staining for 5 min in 0.1% amido black in 5% methanol, 10% acetic acid followed by a 5 min destain in 5% methanol, 10% acetic acid. Unstained nitrocellulose sheets containing the IF proteins were placed in blocking buffer (PBSa, 10% fetal calf serum, 2.5% BSA) for 1 h, rinsed briefly in PBSa, and incubated in either preimmune or immune serum diluted 1:100 with PBSa, 2.5% fetal calf serum, 0.5% BSA. The nitrocellulose sheets were washed with four changes of PBSa over 15 min and then incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Miles-Yeda Research Development Co., Ltd., Rehoveth, Israel) diluted 1:1000 in PBSa, 2.5% fetal calf serum, 0.5% BSA. The sheets were washed with four changes of PBSa over a 15-min period. Proteins that reacted with these antibody preparations were visualized using 0.05% 4-chloro-1-naphthol (Sigma Chemical Co.), 0.01% hydrogen peroxide in PBSa, 15% methanol. In some cases, affinity purification of antisera employing their respective antigens was performed according to the method of Olmsted (12) prior to their use in immunoblotting assays.

Electron Microscopy: Electron microscopic studies of negatively stained protein samples were performed as follows: Paracrystals were fixed with a 1/10 dilution of a 7% glutaraldehyde solution in 50 mM Na phosphate (pH 7.2), and applied to Formvar and carbon-coated copper grids for 1 min. The fixed protein solution was displaced from the grid with several drops of distilled water, stained with 1% aqueous uranyl acetate for 10–20 s, blotted on filter paper to remove excess stain, and air dried. Electron micrographs were taken with a JEOL 100S electron microscope. Magnifications were calibrated using a replica grating.

RESULTS

Intermediate Filament Associated Proteins (IFAPs)

When intact native IF are isolated from fibroblasts (18, 31), a ~55,000-mol-wt polypeptide (vimentin [2] or decamin [31]) is the predominant protein. In the case of BHK-21 cells, a major ~54,000-mol-wt polypeptide (desmin [4, 28]) is also found. Both ~54,000- and ~55,000-mol-wt proteins have been extensively purified, and each is capable of forming homopolymer IF *in vitro*, in the absence of other proteins (19).

In addition to the major IF structural polypeptides, a variety of relatively minor associated proteins (IFAPs) are consistently found in IF preparations isolated from BHK-21, 3T3, and other cultured cell lines considered to be either fibroblasts or epithelial cells (18, 30, 31). Among these are high molecular weight IFAPs (>250,000), whose specific functions remain unknown, although a role for these proteins as IF-IF or IF-microtubule cross-bridging elements has been proposed (15, 18, 32).

Another group of lower molecular weight IFAPs is present in fibroblast IF preparations. These are three to four prominent proteins with apparent molecular weights of 60,000, 65,000, 67,000, and 70,000. These 60,000–70,000-mol-wt proteins co-migrate with proteins found in similar IF preparations from different lines of fibroblasts. The 60,000–70,000-mol-wt polypeptides represent ~10–15% of the total protein in these preparations. Purification of IF structural proteins obtained from BHK-21 and 3T3 cells is achieved by using alternating low-salt/high-salt conditions to effect disassembly and reassembly (18, 32). This results in enrichment of the 60,000–70,000-mol-wt proteins in the low-salt insoluble pellets (Fig. 1). Therefore, this group of proteins is insoluble under conditions in which most of the ~54,000- and 55,000-mol-wt protein is solubilized into small oligomers or protofilaments (18, 30).

Assembly of the 60,000–70,000-mol-wt Proteins into Paracrystals

When native BHK-21 and 3T3 IF preparations are treated with 8 M urea, ~80% of the total protein (including the 60,000–70,000-mol-wt proteins), are solubilized. When these urea extracts are transferred to low ionic strength buffer by rapid gel filtration (see Materials and Methods), no IF are detected by electron microscopy, and the predominant structures observed by negative staining are the 3–5-nm-diam protofilaments reported previously (31). At the protein concentrations and time periods employed in these experiments (<0.5 mg/ml for up to 24 h), very few IF are observed at Na⁺ phosphate concentrations <12 mM in BHK-21 preparations or <15 mM in 3T3 preparations. However, after 24 h at room temperature, paracrystals are often observed in both preparations. These structures are composed of ~2-nm diam filamentous structures which line up in parallel with each other so that their long axes comprise the long axes of the paracrystals. They exhibit an alternating light/dark banding pattern when negatively stained with uranyl acetate. The center-to-

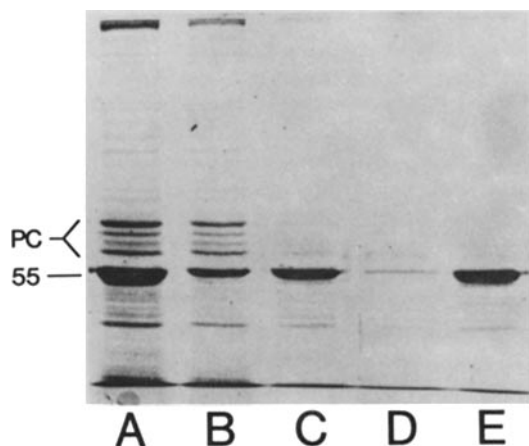


FIGURE 1 Isolation of 3T3 cell IF polypeptides by assembly-disassembly. (A) Freshly isolated IF; (B) 250,000 g pellet after low-salt treatment of dissociated IF; (C) 250,000 g supernatant containing solubilized IF; (D) 150,000 g supernatant remaining after pelleting reconstituted IF; (E) 150,000 g pellet of reconstituted IF. Note that the lower molecular weight IFAPs (PC, 60–70,000K) do not cycle with the major IF structural protein (55,000 mol wt) (lane E). The low-salt insoluble fraction (lane B) is relatively enriched for these IFAPs. For further details, see Materials and Methods.

center spacing between either light or dark staining regions is 37–40 nm; the distance between the edges of the dark regions, where the ~2-nm-diam filaments are visible, is 18–22 nm (Figs. 2 and 3). The paracrystals are often tapered at both ends, but occasionally, there is a “splaying out” at the edges of the paracrystals, more clearly revealing the filamentous fine structure.

No added Ca⁺⁺ or Mg⁺⁺ is required for paracrystal formation. However, 20 μM EDTA prevents the formation of paracrystals, and Ca⁺⁺ or Mg⁺⁺ at concentrations of 0.1–0.5 mM in low ionic strength buffers appears to greatly increase the rate of paracrystal formation. Divalent cation concentrations in excess of 0.5 mM result in rapid IF assembly in preparations from BHK-21 cells. In 3T3 cell preparations, 0.5 mM Mg⁺⁺ or Ca⁺⁺ does not induce rapid IF reassembly. Therefore, buffer conditions were chosen that permitted paracrystal formation in the absence of extensive IF assembly for both the BHK and 3T3 IF systems.

Paracrystal preparations can be pelleted by centrifugation at 10,000 g for 30 min. No paracrystals are found in the supernatant, and the predominant identifiable structures obtained in the pellets are paracrystals. Comparative estimates of protein concentrations in the supernatant and pellet material reveal that ~10–15% of the original native IF protein is pelleted. The predominant components recovered in the pellet are the 60,000–70,000-mol-wt proteins, where they comprise >80% of the total pelleted protein. Nearly all of the protein detectable in the 60,000–70,000-mol-wt region is sedimented with the paracrystals (Figs. 4 and 5). Resolubilization of paracrystals in 8 M urea, followed by gel filtration into low salt and subsequent addition of Na⁺ phosphate and MgSO₄, again frequently results in formation of paracrystals. This provides further purification and enrichment to >90% of the 60,000–70,000-mol-wt polypeptides (Fig. 6).

It should be noted that paracrystal formation is not always obtained when the above procedures are employed. In some experiments, few and sometimes no ordered structures are observed by negative staining, although filamentous aggregates are always observed. In other preparations, paracrystals are the only structures present (Fig. 2). Despite the variability in morphology, the polypeptide composition of the aggregates obtained using this procedure is highly reproducible. In the second cycle of reaggregation, ~80% of the protein is solubilized in 8 M urea. Upon harvesting the aggregates and/or paracrystals in low salt after a “second cycle”, >90% of the protein is recovered. With regard to total amounts of protein, in a preparation from one confluent roller bottle (~2 × 10⁸ cells), which yields ~1 mg of unfractionated IF protein, ~800 μg of total protein is recovered in the first urea extract. About 15% of this, or ~120 μg, is recovered in the first pellet of paracrystals and/or aggregates, and ~60% of this, or ~70 μg, is obtained after the second cycle.

Evidence in Support of the Keratin-like Nature of the 60,000–70,000-mol-wt Paracrystal Proteins

On the basis of our previous biochemical findings that suggested that the 60,000–70,000-mol-wt proteins seen in HeLa cells were keratin-like (30), we decided to determine whether or not this is also the case for the BHK-21 and 3T3 paracrystal proteins. In the case of HeLa, evidence of the keratin-like nature of the 60,000–70,000-mol-wt proteins was based upon their amino acid compositions and their cross-

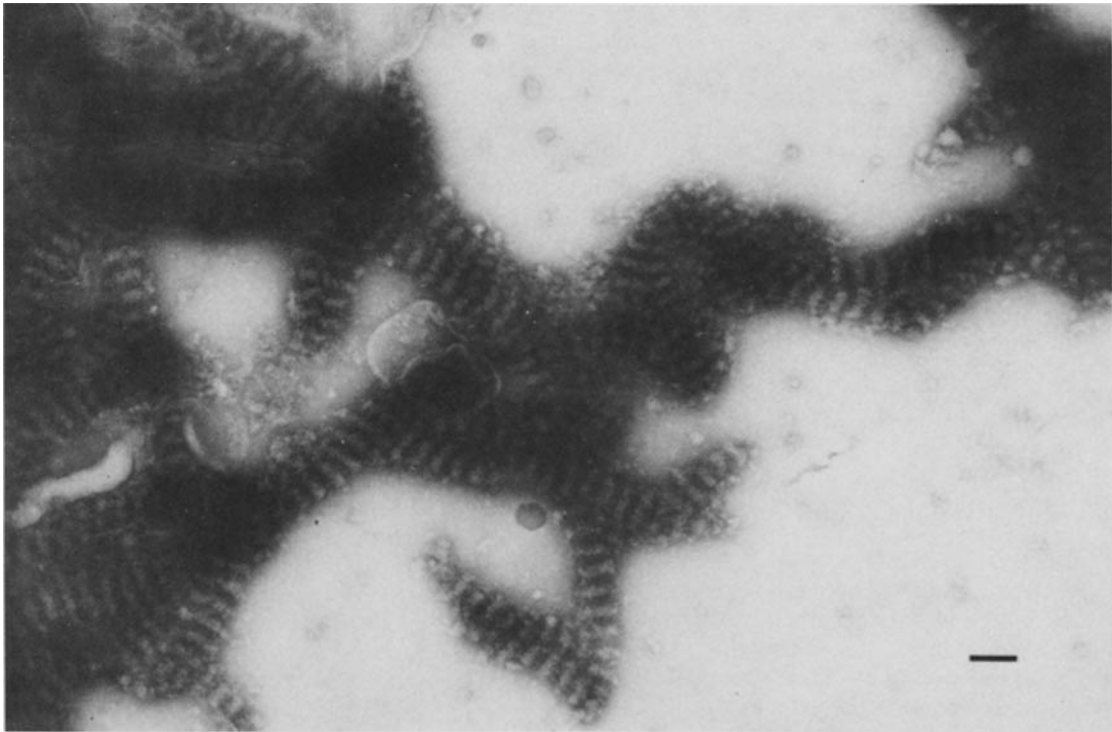


FIGURE 2 Negatively stained (1% uranyl acetate) paracrystals from mouse 3T3 cell preparations. Bar, 0.1 μm . $\times 60,000$.



FIGURE 3 Negatively stained (1% uranyl acetate) paracrystal from BHK-21 cell preparation. Bar, 0.1 μm . $\times 150,000$.

reactivity with certain antibodies directed against purified keratin (reference 30 and Whitman-Aynardi, M., P. Steinert, and R. Goldman, unpublished observations).

Purified paracrystal proteins from BHK-21 and 3T3 IF preparations have been subjected to screening by immunoblotting (see Materials and Methods), to determine whether any of the paracrystal proteins cross-react with various keratin antibody preparations, as well as BHK-21 55,000-mol-wt IF antiserum. Specific cross-reactions with one or more paracrystal proteins can be seen with mouse epidermal keratin (K1) antiserum (Figs. 7 and 8). Antiserum directed against BHK-21 55,000-mol-wt protein shows a reaction with its respective antigen, but not with the paracrystal bands. Affinity

purification by the method of Olmsted (12) of the K1 antibody also resulted in a specific reaction with the 70,000-mol-wt paracrystal band, but did not appear to react with other paracrystal proteins.

The amino acid composition of each of the four BHK-21 paracrystal proteins and two of the 3T3 (60,000 and 70,000 mol wt) proteins has been determined (Materials and Methods). The data are presented in Table I. By comparing the data, it is apparent that all of the paracrystal proteins are very similar to the keratins. This is especially evident if one compares the glycine content of the various proteins shown in Table I. The keratins are distinguished by their significantly elevated glycine contents when compared with much lower

FIGURE 4 Enrichment of paracrystal proteins from BHK-21 cells. (A) 250,000 g 8 M urea extract of BHK freshly isolated IF; (B) 10,000 g supernatant remaining after pelleting paracrystals; (C) 10,000 g paracrystal pellet. The major 55,000-mol-wt polypeptide (55) and the paracrystal polypeptides (PC) are indicated. For further details of paracrystal isolation, see Materials and Methods.

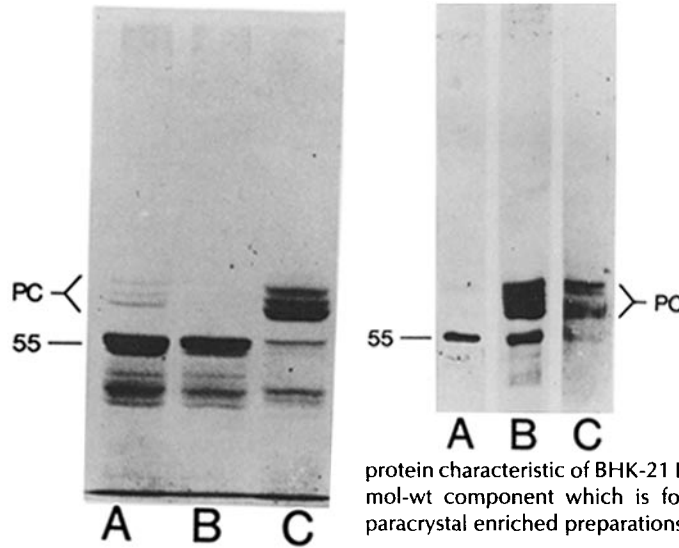


FIGURE 7 Immunoblotting of an enriched BHK-21 paracrystal preparation. This preparation was subjected to SDS PAGE and transferred to nitrocellulose, and then strips were cut out and used for staining with amido black (Lane B) or treated with either BHK-IF antiserum (Lane A) or mouse epidermal keratin antiserum (Lane C). Major paracrystal proteins (PC) seen with amido black in B react primarily with keratin antiserum (Lane C). The antiserum directed against the major structural protein characteristic of BHK-21 IF reacts primarily with the 55,000-mol-wt component which is found in reduced amounts in the paracrystal enriched preparations (Lane A).

FIGURE 5 Enrichment of paracrystal proteins from mouse 3T3 cells. (A) 250,000 g 8 M urea extract of 3T3 freshly isolated IF; (B) 10,000 g supernatant remaining after pelleting paracrystals; (C) 10,000 g paracrystal pellet. The major 55,000-mol-wt polypeptide and the paracrystal polypeptides (PC) are indicated. For further details of paracrystal isolation, see Materials and Methods.

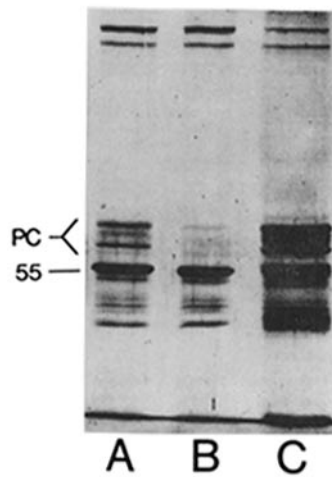
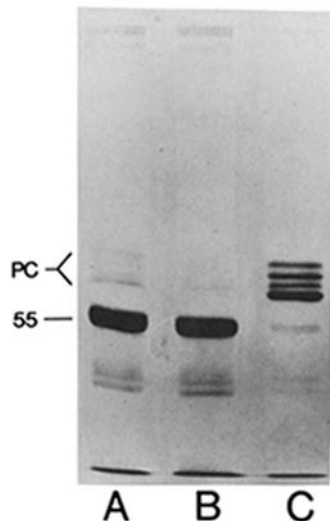


FIGURE 8 Immunoblotting of a native IF preparation obtained from 3T3 cells. Lane A, amido black stain of nitrocellulose strip after transfer. Two paracrystal (PC) proteins have transferred efficiently, the others have not. The 55,000-mol-wt major structural protein is also present. Lane B shows a positive reaction with one of the major paracrystal proteins after treatment with mouse epidermal keratin (K1) antiserum. Lane C shows the reaction with the BHK 55,000-mol-wt IF antiserum. Lane D is a preimmune control.

FIGURE 6 Further purification of 3T3 cell paracrystal polypeptides by cycles of paracrystal assembly-disassembly. (A) 250,000 g 8 M urea extract of freshly isolated IF; (B) 10,000 g supernatant remaining after harvesting paracrystals from A; (C) paracrystal polypeptides obtained in the second 10,000 g pellet of twice-cycled paracrystals. The 55,000-mol-wt polypeptide and paracrystal polypeptides (PC) are indicated. For further procedural details, see Materials and Methods.



($\pm 20\%$), and 67,000- and 70,000-mol-wt polypeptides exhibited 30% ($\pm 20\%$) α -helix.

DISCUSSION

A considerable number of studies and review articles have been published over the past few years that suggest that the keratins are a specific group of epithelial cell proteins that are not found in other cell types. Most of these studies have been based primarily on data obtained by indirect immunofluorescence, in which epithelial cells and tissues react specifically with keratin antibodies, but not other cells and tissues (11, 32). However, negative immunofluorescence observations made in situ could be misleading owing to many types of potential artifacts, including, for example, improper fixation and subsequent solubilization of certain antigens, denaturation of antigenic sites, inaccessibility of antigenic determinants because of the three-dimensional configuration of proteins in situ, and the presence of small numbers of antigenic sites in certain cell types which results in low (difficult to detect) levels of fluorescence. On the basis of these and other potential pitfalls that stem from the exclusive use of fluorescent antibody methods, we have attempted to determine, using biochemical and immunochemical techniques, whether

numbers seen in the major IF structural proteins that characterize fibroblastic cells (also see reference 32).

Percentage α -helix determinations of the BHK-21 paracrystal polypeptides are also consistent with their keratin-like properties. The α -helical content of electrophoretically purified 3T3 60,000- and 65,000-mol-wt polypeptides was 35%

TABLE I
Amino Acid Compositions of IF Subunits and Paracrystal Proteins

Amino acid	Mouse epidermal keratin IF 65K*	Mouse 3T3 fibroblast				BHK-21 Fibroblast				
		IF		Paracrystal		IF		Paracrystal		
		55K [‡]	60K	70K	55K	60K	65K	67K	70K	
Asp	8.36	8.7	4.26	7.74	9.1	6.45	6.29	6.03	5.89	
Thr	3.21	4.8	4.05	4.05	4.6	3.99	4.21	4.47	4.82	
Ser	12.29	9.6	17.63	18.47	9.5	17.17	17.56	17.83	18.04	
Glu	12.34	14.8	16.39	15.62	15.2	15.22	14.92	13.25	10.69	
Pro	1.52	0.9	2.13	1.61	0.7	1.34	1.06	0.97	0.76	
Gly	22.43	10.0	21.82	19.92	9.8	21.07	23.62	24.92	27.02	
Ala	6.44	6.5	6.10	5.39	6.4	4.96	4.54	4.78	4.95	
½Cys	0.67 [§]	0.7 [§]	0.29 [§]	0.32 [§]	0.8 [§]	0.71 [¶]	0.75 [¶]	0.74 [¶]	0.67 [¶]	
Val	3.29	5.5	4.35	4.43	5.7	2.18	2.08	2.23	3.19	
Met	2.73	2.1	1.03	0.92	2.2	1.22	1.29	1.17	1.49	
Ile	3.19	3.4	2.81	2.82	3.5	2.89	1.93	2.37	2.50	
Leu	5.96	11.3	7.30	6.68	11.6	8.06	7.08	6.98	7.04	
Tyr	1.83	2.9	2.05	2.19	2.8	2.51	2.53	2.31	2.02	
Phe	3.62	2.6	1.74	1.81	2.6	2.75	3.20	2.98	2.0	
His	1.2	1.7	1.47	1.53	1.8	0.94	1.04	1.03	1.21	
Lys	5.44	6.0	4.07	3.76	6.0	4.91	3.53	3.39	3.26	
Try	0.63	0.37	0.22	0.22	0.38	ND	ND	ND	ND	
Arg	4.75	8.5	2.16	2.48	8.2	4.16	4.13	4.36	4.60	

All data are expressed as residues/100 residues. 65K, 65,000-mol-wt protein; 55K, 55,000-mol-wt protein; etc. ND, not determined.

* From reference 20.

† From reference 32.

‡ Measured as described in reference 32.

¶ Measured as cysteic acid as described in reference 29.

or not keratin-like proteins are present in continuously cultured cells normally considered to be fibroblasts. It should be emphasized that cells such as BHK-21 and 3T3 are considered to be fibroblasts because of their overall shape.

In this paper we have presented immunological and biochemical data that indicate the presence of keratin-like proteins in two types of mammalian cell lines, hamster BHK-21 and mouse 3T3. These cells are normally considered to be devoid of keratin (3). These keratin-like proteins also appear to be present in other fibroblast and epithelial cell lines (unpublished observations), and they co-isolate with native IF. However, in fibroblast preparations they do not co-purify with IF during cycles of assembly/disassembly *in vitro*. Furthermore, these proteins form aggregates or paracrystals under conditions in which the major IF structural protein(s) remain soluble in the form of oligomers or protofilaments (30).

All keratin and other IF proteins thus far examined possess a specific secondary structure: two nearly equal-sized α -helical regions, and three non- α -helical regions (5, 23, 24, 32). The dimensions of the paracrystals formed from the 60,000–70,000-mol-wt proteins are consistent with this secondary structure, i.e., the length of the dark-staining paracrystal bands is 18–22 nm, very close to the length of each α -helical domain in IF polypeptides, as determined by calculation from sequence data (5, 23), as well as by direct measurement of proteolytically derived α -helical fragments (5, 17, 24). Direct determination of the percent α -helix of each of the paracrystal proteins lends further support to the idea that these proteins possess the secondary structure expected for IF proteins.

While the precise packing arrangement of polypeptides in the paracrystals remains to be elucidated, it is likely that the light/dark banding pattern is derived from the alignment of ~2-nm-diam helical regions of adjacent subunits (17, 24, 32) to form the dark bands of the paracrystal, while the light

regions could be composed of the closely associated nonhelical domains on adjacent subunits. Thus, the structure of the paracrystals also suggests that they are composed of IF polypeptides. Taken together with the immunological and biochemical data, these structural results strongly suggest that BHK-21 and 3T3 cells contain keratin-like proteins.

It is our belief that conclusive identification of IF structural proteins must include evidence that they are capable of forming IF *in vitro*. Despite persistent attempts to assemble the 60,000–70,000-mol-wt paracrystal proteins into IF, we have not been able to demonstrate that these proteins form IF *in vitro*. However, this behavior is not totally unexpected if these are keratin-like proteins. Epidermal keratin polypeptides form IF *in vitro* only as heteropolymers (i.e., two or more specific polypeptides are required to form a multi-chain coiled-coil complex, the penultimate “subunit” of all IF thus far characterized [5, 21–23, 31]). It is possible that fibroblasts do not contain the other keratin polypeptides necessary for the paracrystal proteins to form IF. Indeed, preliminary immunofluorescence evidence suggests that these proteins are not organized into a cytoplasmic filamentous pattern characteristic of IF but rather exhibit a perinuclear location similar to that seen with nuclear lamin antibodies (6) (unpublished observations). We are currently investigating whether these keratin-like proteins may be involved in cytoplasmic IF association with or anchorage to the nucleus (9), and whether or not they are related to the nuclear lamins (6). If this proves to be the case, then one would predict that similar keratin-like proteins may be present as a nucleus-associated component in all cells. However, further work is required to determine the exact subcellular localizations and functions of these proteins, and to determine more precisely how these proteins are related to those that form the keratin-containing cytoplasmic arrays of tonofilaments which are characteristic of epithelial cells.

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Note Added in Proof: Preliminary data supporting the possibility that the IFAPs described in this paper are nuclear associated was presented at the 23rd Annual Meeting of the American Society for Cell Biology and an abstract has been published (Goldman, R. D., A. E. Goldman, J. Jones, G. Maul, H. Yang, and R. Zackroff, 1983, *J. Cell Biol.*, 97 [5, Pt. 2]: 221a).

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