Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 4534–4538, August 1980
Biochemistry

Intermediate filaments of baby hamster kidney (BHK-21) cells and bovine epidermal keratinocytes have similar ultrastructures and subunit domain structures

(10-nm filaments/methionine cleavage/tryptophan cleavage/α-helix-enriched particles)

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Communicated by David R. Davies, May 2, 1980

ABSTRACT **Structural features of the intermediate filaments (IF) of bovine epidermal keratinocytes (keratin filaments) and of baby hamster kidney (BHK-21) cells were studied to define the molecular basis of the similarities and differences in their properties. Purified subunits of the IF were specifically cleaved at their methionine and tryptophan residues. Peptide maps indicated that the IF subunits of BHK-21 cells were similar but not identical to each other and quite different from each of the keratin IF subunits. Intact IF were subjected to limited tryptic digestion, and structurally identical α-helix-enriched particles were released. Their properties indicated these IF were composed of a similar three-chain unit which contained regions of coiled-coil α-helix interspersed with regions of non-α-helix. These two types of experiments permitted the construction of subunit domain maps which revealed a common structure: all subunits possessed two α-helical domains of the same size that were adjoined by non-α-helical domains of variable size. We propose that the reported solubility and immunological differences in these IF and perhaps those of other types of cells are due largely to variations in the size, configuration, and amino acid sequence of the non-α-helical regions of the subunits in the IF.**

Ten-nanometer or intermediate filaments (IF) are ubiquitous constituents of the cytoskeleton of higher eukaryote cells. They have been isolated from various different cells and tissues either as intact filaments or as soluble subunits. They appear to differ in their solubility and immunological properties and in their subunit complexity and molecular weight (1–3). Based largely on immunofluorescence data, four or five distinct subclasses of IF have been described in different cells (1–3), and some cells are thought to contain at least two immunologically distinct types of IF (4–7). However, classifications based on immunological data alone may be premature. The techniques used for the preservation and fixation of cells are of critical importance because the subsequent antibody-staining protocols can provide variable results (8, 9). Also, the observations may vary depending on whether the antibody was elicited against intact IF or a purified IF subunit (9). Therefore, in view of these potential hazards, more rigorous structural and biochemical studies are required to define the IF of different cells. Such information is still limited, but studies on the IF of keratinocytes, cells of mesenchymal origin, and neuronal tissues have revealed some common properties. All of these IF are long tubes of protein 8–10 nm in diameter and contain highly α-helical α-type fibrous proteins of the k-m-e-f class (2, 10–13).

In the study reported here, we used specific chemical and structural techniques on either intact IF or purified subunits of the IF of baby hamster kidney (BHK-21) cells and bovine epidermal keratinocytes. We show that the IF of these cells are composed of a common three-chain structural unit. Their subunits possess α-helical domains of the same size but non-α-helical domains of varying size, features which may account for the observed properties of these IF.

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MATERIALS AND METHODS

Isolation of Filaments and Subunits. BHK-21 cells were propagated in culture and their IF were isolated as intact filament caps (11). The filaments were then purified by repeated cycles of assembly/disassembly *in vitro* (12, 14). We used this procedure to remove nonfilament proteins and to ensure that the proteins used in this study were functional IF subunits. On two-dimensional polyacrylamide gels, the subunits consisted of a major protein component, decamin (14), of $M_r \approx 55{,}000$ and two smaller more basic proteins each of $M_r \approx 54{,}000$. The apparent molecular weight difference of these two subunits was <500 and their pI values differed from each other and from decamin by about 0.1. They were termed subunits a (larger) and b (smaller, most basic), respectively. Bovine epidermal keratin IF consist of at least 10 different subunits of M_r 47,000–68,000, the quantitatively major ones of which are termed 1c, 1a, 3, 4, and 5 (15, 16). Filaments were reconstituted *in vitro* from separated and purified subunits to give compositions of 1c/3/5 and 1a/4/5 in molar ratios of 1:1:1 (16, 17).

Specific Cleavage Procedures. Purified IF subunits (1 mg/ml in 70% formic acid) were cleaved at their methionine residues with an equal weight of CNBr for 2 days at 23°C. *N*-Bromosuccinimide (NBS) (18) and *o*-iodosobenzoic acid (19) were used to cleave the subunits at their tryptophan residues.

Analytical Procedures. Protein was estimated by the method of Bramhall *et al.* (20). Polyacrylamide gel electrophoresis on one-dimensional gels containing $NaDodSO_4$ was performed on either 10–18% acrylamide gradients as 1.5-mm-thick slabs by the method of Laemmli (21) or 12% tube gels with use of maximal unstacking parameters (15, 22). Molecular weights were estimated by construction of Ferguson plots (15). Isoelectric focusing was done on 7% acrylamide gels in the pH ranges of 3.5 to 10 or 3.5 to 8 (23). Two-dimensional gel electrophoresis was done by the method of O'Farrell (24). The intact subunits of the IF and the NBS-cleavage products and the particle 1 peptides of certain subunits were separated by preparative gel electrophoresis (15) and their homogeneity was confirmed on two-dimensional gels. The *N*-acylated amino termini (*N*-acetylserine and *N*-acetylalanine) were isolated in

Abbreviations: IF, intermediate filament(s); NBS, *N*-bromosuccinimide.

yields of 0.5–0.8 mol/mol after total enzymic digestion (15). Carboxyl-terminal amino acids were released from the subunits with carboxypeptidase Y (Calbiochem) (1 unit/nmol of protein) in 6 M urea/0.1% $NaDodSO_4$/0.05 M phosphate/0.05 M NaCl, pH 5.5 or 8.0, in yields of 0.2–1.0 mol/mol. α-Helix contents were estimated by two methods, optical rotatory dispersion and circular dichroism (12, 18), because the α-helix content assigned to a protein species may vary with the model used. In this work, the two methods yielded estimates that were within ±10% of each other.

Isolation and Characterization of α-Helix-Enriched Particles. Epidermal keratin and BHK-21 IF were dispersed in 1 mM sodium tetraborate/1 mM KCl, pH 9.2. Under these conditions, the former become shorter (18) and the latter disassemble into protofilamentous units (14). The proteins were digested with trypsin (Sigma, type III) for up to 1 hr at 23°C (18, 25). The released α-helix-enriched particles were then separated by chromatography on Sepharose 6B for physicochemical characterization (18, 25, 26).

RESULTS

Specific Chemical Cleavage of IF Subunits. When resolved by isoelectric focusing, the CNBr peptides of the three subunits of the BHK-21 IF were similar, but there were significant differences in the pI values of some peptides (Fig. 1). The peptides of the major bovine epidermal keratin IF subunits were quite different from each other and from those of the BHK-21 IF. However, all subunits contained several acidic and basic CNBr peptides, which suggests that there are distinct acidic and basic domains in the subunits. Preliminary characterization of these peptides has revealed that only the acidic species are α-helical (unpublished data).

Chemical cleavage of the subunits at their tryptophan residues, by any of the currently available techniques, resulted in only slight reaction. Cleavage with NBS yielded the most reproducible results; for each subunit, two large and several smaller peptides were obtained, as well as uncleaved protein (Fig. 2). The two largest peptide species, designated W-1 and W-2, of the BHK-21 and keratin IF subunits were separated and characterized (Table 1; not all data shown). In all cases, they were found to have the *N*-acylated amino-terminal residue of the intact subunit; that is, they arose by cleavage at two tryptophan residues located toward the carboxyl terminus. Peptides W-1 and W-2 contained all of the α-helix of the intact subunits, indicating the tryptophan residues were located in non-α-helical regions.

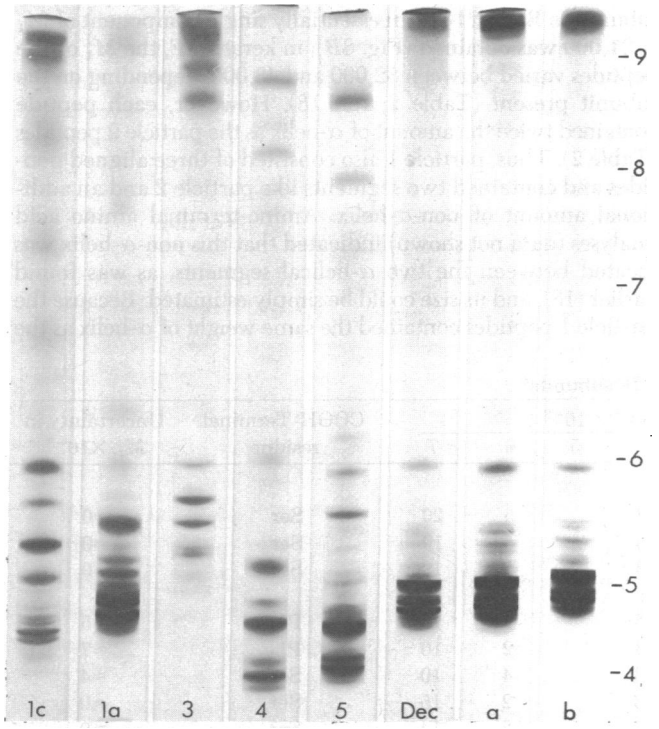

FIG. 1. CNBr peptides of IF subunits. The approximate pH values along the isoelectric-focused gels are indicated. The gels were aligned by a band of bromophenol blue. 1c, 1a, 3, 4, and 5 are the major bovine epidermal keratin IF subunits; decamin (Dec), a, and b are the subunits of the IF of BHK-21 cells.

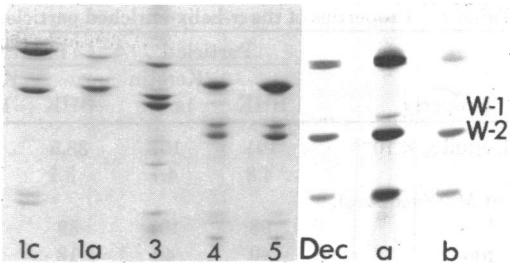

FIG. 2. NBS peptides of IF subunits. In each lane, the upper band is the uncleaved subunit. The two larger cleavage products are termed peptides W-1 and W-2.

Isolation and Characterization of α-Helix-Enriched Fragments. The BHK-21 and keratin IF were subjected to limited tryptic digestion as done previously with bovine (18) and human (25) epidermal keratin and related (26) filaments, and two peaks of protein highly enriched in α-helix were obtained (Fig. 3, data for BHK-21 IF). However, the total recoveries of α-helix from the BHK-21 IF did not exceed 40%, whereas the yields from keratin IF exceeded 80% (see refs. 18

Table 1. Properties of the products of cleavage at tryptophan

Subunit or fragment	$M_r \times 10^{-3}$	Average α-helix content, %	Total M_r of α-helix $\times 10^{-3}$	NH_2-Terminal residue
		From BHK-21 IF		
Decamin:				
Unreacted	55	42	23.1	*N*-AcAla
Uncleaved	55	43	23.7	
W-1	35	68	23.8	
W-2	30	75	22.5	
a:				
Unreacted	54	44	23.7	*N*-AcAla
Uncleaved	54	42	22.7	
W-1	34	70	23.8	
W-2	29	75	21.8	
		From keratin IF		
1a:				
Unreacted	58	40	23.2	*N*-AcSer
Uncleaved	58	42	24.4	
W-1	48	48	23.0	
W-2	46	50	23.0	
5:				
Unreacted	48	48	23.0	*N*-AcSer
Uncleaved	48	50	24.0	
W-1	34	70	23.8	
W-2	32	75	24.0	

The M_r values ($\times 10^{-3}$) of the other intact subunits and their W-1 and W-2 peptides were, respectively: BHK-21 IF subunit b, 54, 33, and 29; keratin subunit 1c, 65, 50, and 48; subunit 3, 54, 44, and 40; and subunit 4, 50, 35, and 33.

Table 2. Properties of the α-helix-enriched particles

Property	Particle 1 BHK	Particle 1 Keratin 1a/4/5	Particle 2 BHK	Particle 2 Keratin 1a/4/5
M_r (sed. equil.), × 10^{-3}	101	102	38.5	40
$s^0_{20,w}$	4.8	4.7	3.1	3.1
Apparent M_r (Svedberg), × 10^{-3}	98	101	39	40
Length, nm	40	40	18	18
Average α-helix content, %	75	73	>85	>85
Total weight of α-helix, × 10^{-3}	74.6	74.1	≥33	≥35
M_r of peptides on dissociation with $NaDodSO_4$, × 10^{-3} *	31	1a: 39 4: 33 5: 32	13	13
Total M_r of α-helix in isolated particle 1 peptides, × 10^{-3} *†	25	1a: 25 4: 23 5: 24		

* M_r of particle 1 peptides derived from keratin subunits 1c and 3 of 1c/3/5 filaments were 43,000 and 36,000, respectively; they both contained about M_r 25,000 of α-helix.

† α-Helix contents of the dissociated particle 2 peptides were not estimated. Based on the α-helix content of the intact particle 2, the peptides probably contain about M_r 12,000 of α-helix. This value is consistent with the data of Table 1 and with the actual size of the known sequence of α-helical regions of wool (27, 28). Thus, particle 2 is probably not completely α-helical; there may be non-α-helical discontinuities along the α-helical region or short non-α-helical sections on the ends.

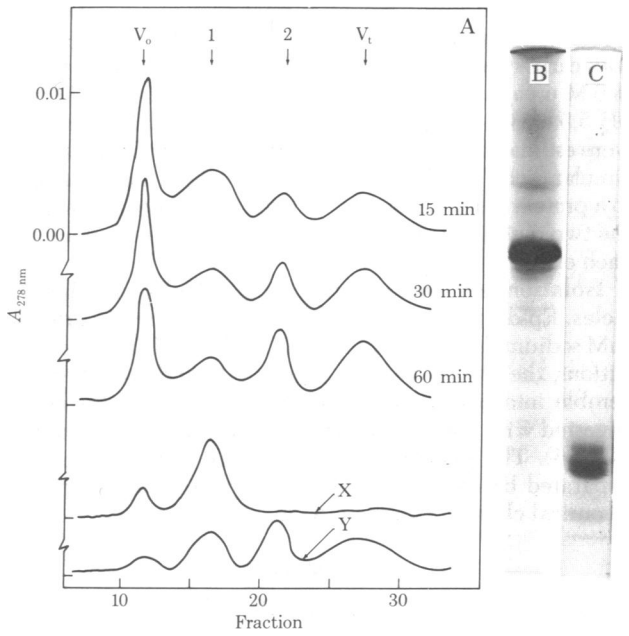

FIG. 3. Isolation of the α-helix-enriched particles from IF of BHK-21 cells. (*A*) Aliquots containing ≈0.5 mg of protein were removed from the tryptic digestions at the indicated times, terminated with trypsin inhibitor, and chromatographed on Sepharose 6B (18). A sample from peak 1 at t = 15 min of a similar experiment was removed for rechromatography (curve X) or redigestion (30 min) and then rechromatography (curve Y). (*B* and *C*) Samples from peak 1 (t = 15 min) and peak 2 (t = 60 min), respectively, were dissociated in $NaDodSO_4$ for gel electrophoresis on maximally unstacked gels.

and 26). In all cases, the material of peak 1 could be redigested to yield mostly peak 2. After negative staining with uranyl acetate, peaks 1 and 2 contained particles of fairly uniform width (≈20 Å) and of mean (± SD) lengths 400 ± 45 and 180 ± 20 Å, respectively (data not shown; ref. 18). Other physicochemical properties of the particles, summarized in Table 2, demonstrate that the particles of keratin and BHK-21 IF were identical and indistinguishable from those isolated previously (18, 25, 26). The particle 2 species (apparent M_r, ≈39,000; α-helix content, >85%) dissociated in $NaDodSO_4$ solution to peptides of M_r ≈ 13,000 (Fig. 3*C*)—that is, about one-third of their intact molecular weights. Consideration (18) of their α-helix contents and lengths leads to the conclusion that these particles consist of three peptides aligned side-by-side to form a rod-shaped coiled-coil of α-helix. This structure or segment within the filaments is responsible for the α-type x-ray diffraction pattern of the filaments (12, 18).

The low yield of particle 2 from BHK-21 IF suggests there are regions of discontinuity in the coiled-coil which permit degradation during the isolation procedures. The particle 1 species (apparent M_r, ≈100,000) dissociated in $NaDodSO_4$ solution to peptides of about one-third of their intact molecular weight, but the size of these peptides varied with the filament subunit: in BHK-21 IF, an essentially single component of M_r ≈ 31,000 was obtained (Fig. 3*B*); in keratin IF, the M_r of the peptides varied between 32,000 and 43,000, depending on the subunit present (Table 2; ref. 18). However, each peptide contained twice the amount of α-helix as the particle 2 peptides (Table 2). Thus, particle 1 also consisted of three aligned peptides and contained two segments like particle 2 and an additional amount of non-α-helix. Amino-terminal amino acid analyses (data not shown) indicated that this non-α-helix was located between the two α-helical segments, as was found earlier (18), and its size could be simply estimated. Because the particle 1 peptides contained the same weight of α-helix as the

Table 3. Domains of IF subunits*

Protein	M_r × 10^{-3}	NH_2-Terminal residue	M_r of domains,† × 10^{-3} 1	2	3	4	5	6	7	COOH-Terminal residue	Uncertainty in M_r, ×10^{-3}
BHK-21											
Decamin	55	*N*-AcAla		13	5	13		5	20	Ser	≈0
a	54	*N*-AcAla		13	5	13		5	19	Ser	≈0
b	54	*N*-AcAla		13	5	13		5	19	Ser	≈0
Keratin:											
1c	65	*N*-AcSer		13	17	13		2	15	Phe	≈5
1a	58	*N*-AcSer		13	13	13		2	10	Phe	≈7
3	54	*N*-AcSer		13	10	13		4	10	Ser	≈4
4	50	*N*-AcSer		13	7	13		2	15	Ser	≈0
5	48	*N*-AcSer		13	6	13		2	14	Ser	≈0

* See Fig. 4 for map.

† See map for identification of domain (Fig. 4). Calculation of domain sizes: domains 2 and 4, from particle 2, each α-helical sequence was the same, ≈13,000 (Fig. 3*C*); domain 3, from the difference between the size of the particle 1 peptides of each subunit and domains 2 plus 4; domains 6 and 7, from the difference in size between the intact subunit and its respective W-1 and W-2 peptides; domains 1 and 5, not known.

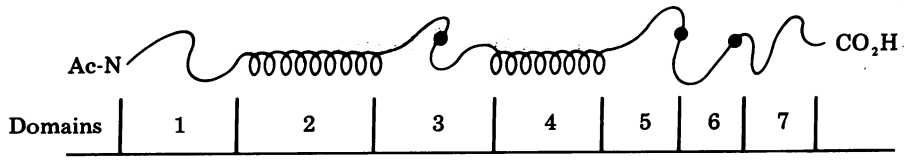

FIG. 4. Domain map of IF subunits. ●, Tryptophan residue.

entire IF subunits (compare Tables 1 and 2), the particle 1 peptides and the W-2 peptides contain the same overlapping sequences.

DISCUSSION

Common Domain Structure of the IF Subunits. The observations on the properties of the W-1 and W-2 peptides of the IF subunits and on the size of the particle 1 peptides permitted the construction of the subunit domain maps illustrated in Fig. 4 and Table 3. There are marked similarities in chemistry, distribution of α-helix, and overall subunit structure. The two α-helical domains, domains 2 and 4, were each $\approx$180 Å long, had $M_r \approx$ 13,000, and were located toward the amino terminus of the subunit. They were separated by the non-α-helical domain 3 which varied widely in size among different subunits. In addition, each subunit contained a region of non-α-helix at its carboxyl terminus of varying size that represented about one-third of the intact subunit. This was resolved into domains 6 and 7, delineated by two tryptophan residues. Determination of the sizes of the presumably small domains 1 and 5 will have to await amino acid sequence analyses. It is noteworthy that the α-helical sections of wool keratin filaments are also 150–170 Å long and $\approx$12,000 in M_r (27–29). Thus, in view of the marked similarities in ultrastructure and chemistry of the IF of different cell types so far studied (2, 10–13), it is likely this subunit structure of α-helical domains of similar size separated by non-α-helical domains of variable size will be common to most if not all IF.

The presence of common terminal residues suggests that the smaller subunits of the IF of BHK-21 cells are not degradation products of the major subunit (decamin), but all three yielded similar peptide maps (Figs. 1 and 2; ref. 7). Two reports have suggested that the smaller IF subunits in BHK-21 cells are desmin, the IF type of smooth muscle (6, 7). If BHK-21 cells contain the two types of IF, then it is clear that they are structurally and chemically similar. Their proposed immunological differences must be due to subtle conformational or amino acid sequence variations. The a and b presumptive-desmin subunits differ from one another in that subunit b contains one or two fewer serine phosphate residues per mol than does subunit a (unpublished data).

Common Three-Chain Structural Unit of IF. The isolation of the three-chain particle 1 from epidermal keratin and BHK-21 IF, the peptides of which contained all of the α-helix of and about two-thirds of the mass of the intact subunits, indicates that the IF themselves consist of a repeating three-chain structural unit (18, 25, 26). The IF of BHK-21 cells can be dispersed at low ionic strength and epidermal keratin IF can be dispersed with a citrate buffer (prekeratin) to particles of size comparable to this three-chain unit (11, 14, 18). In the proposed model (Fig. 5), three subunit chains are aligned in approximate register. The adjacent α-helical sections form two discrete three-chain coiled-coil segments each $\approx$180 Å long and are separated by regions of non-α-helix. The overall length of the three-chain unit is 470–480 Å, based on electron microscopic data from both keratin and BHK-21 IF (unpublished results) and x-ray diffraction data from wool keratin filaments (29). Also, the morphological similarity and thus common symmetry of the IF so far studied logically requires that the three-chain units be of the same length. That is, the lengths of three-chain units of keratin IF of subunit composition 4/5/5 ($M_r \approx$ 148,000) or 1c/3/3 ($M_r \approx$ 175,000) and of BHK-21 IF ($M_r \approx$ 165,000) are independent of the molecular weights. From the simple calculations outlined previously (18), this means that the non-α-helical regions must assume diameters of 25–35 Å, which are considerably more than the diameter of the rod-shaped α-helical segments ($\approx$20 Å).

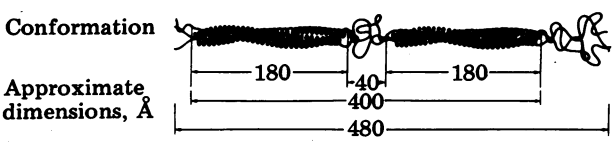

FIG. 5. Model of the three-chain structural unit of IF. The three-chain coiled-coil α-helical segments ($\approx$180 Å long) correspond to particle 2, and the region containing both segments ($\approx$400 Å long) corresponds to particle 1. Thus, the non-α-helix of domain 3 is $\approx$40 Å long and that of domains 1 plus 5 to 7 is perhaps $\approx$80 Å long. An alternative model in which the three adjacent subunits are partially folded (18) so that two of the three α-helices for one segment are contributed by one subunit cannot be excluded.

The entire IF obviously consist of many such units associated both end-to-end and side-by-side in an as yet undefined manner. However, the available evidence from wool keratin filaments (29) indicates that the α-helical segments comprise the basic framework of the filaments. The non-α-helical regions occupy interstitial spaces and also partly project beyond the wall of the filament where they probably interact with the environment. Because the keratin and BHK-21 IF differed from one another primarily in the size of their non-α-helical regions (Table 3), we propose that differences in the size, configuration, and amino acid sequence of these non-α-helical regions may account for the reported differences in solubility, antigenicity, and other properties of the IF of different cells. It is also noteworthy that, even though of similar size, the α-helical sections display significant amino acid sequence variability (Fig. 1; refs. 27 and 28), which may further distinguish the subunits of the IF.

Recently, it was suggested that IF subunits are analogous to IgG molecules in having constant and variable domains (3). This hypothesis is at best an oversimplification: the heavy and light chains of different IgG molecules are all similar in size, but the subunits of the IF of different cells have molecular weights varying between 47,000 and 70,000 (1–3). Previous (18) and present findings demonstrate that there are domains of constant and variable *size* in different IF subunits.

We thank Dr. E. Appella for assistance with the amino-terminal amino acid analyses. This work was supported in part by a grant (to R.D.G.) from the National Science Foundation.

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