Epidermal Keratin Filaments Assembled In Vitro Have Masses-per-Unit-Length That Scale According to Average Subunit Mass: Structural Basis for Homologous Packing of Subunits in Intermediate Filaments

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ABSTRACT We have used scanning transmission electron microscopy to elucidate the question of how intermediate filament (IF) subunits of widely differing mass can all form morphologically similar IF. From scanning transmission electron micrographs, the distributions of mass were determined for three types of epidermal keratin IF reassembled in vitro from mixtures of subunits with substantially different masses, viz., "light" and "heavy" human keratins with $\langle M_i \rangle = 50,000$ and $56,000$, respectively, and mouse keratins of $\langle M_i \rangle = 63,000$. Their principal assembly products were found to average 22, 25, and 29 kdalton/nm, respectively. These densities, which correspond to immature "minimal form" IF (Steven, A. C., J. Wall, J. Hainfeld, and P. M. Steinert, 1982, Proc. Natl. Acad. Sci. USA., 79:3101-3105), are directly proportional to the average subunit masses. The human keratin IF (but not those of mouse) also contained minor amounts (15-20%) of more massive polymers averaging 33 and 35 kdalton/nm, respectively, which probably represent mature IF. Taken together with earlier results on IF of other subclasses, these results indicate that the average linear density of IF scales according to the average mass of their constituent subunits, both for "minimal form" and for mature IF. As underlying mechanism for this homology, we propose that the fundamental building-blocks of all these IF contain a common structural element whose packing within the various IF is likewise conserved and which specifies the overall structure. The variable amounts of mass in the non-conserved moieties account for the observed proportionality. This scheme fits with amino acid sequence data for several IF subunits that have revealed, as a likely candidate for the common element, an essentially conserved $\alpha$-helical domain, contrasting with the highly variable sequences of their non-$\alpha$-helical terminal domains.

By immunological and biochemical criteria, the cytoskeletal intermediate filaments (IF) of eucaryotic cells have been categorized into five subclasses: mesenchymal vimentin, muscle desmin, glial filaments, neurofilaments, and the epithelial keratins (1-5). Of these, keratin IF are the most complex in the sense of subunit diversity, with molecular weights ranging from 40,000 to $\sim 70,000$. Moreover, unlike other IF, the keratins seem to be obligate heteropolymers: no keratin subunit has yet been shown to be individually competent for filament assembly in vitro, whereas many combinations of subunits co-assemble efficiently. Nevertheless all native and reassembled IF show certain structural properties, including their apparent diameters (8-12 nm) and rather bland morphologies as determined by conventional electron microscopy (6, 7). Also, x-ray diffraction patterns exhibiting reflections characteristic of coiled-coil $\alpha$-helices have been obtained for native wool and porcupine quill and for worm giant axons, as well as for several types of IF reassembled in vitro (8-13). Beyond these general properties, however, many more specific

1 Abbreviations used in this paper: IF, intermediate filament(s); STEM, scanning transmission electron microscopy.
questions concerning the structural organization of subunits within IF remain to be settled. One problem applies in particular to the keratins: while the wide range of subunit combinations that may be co-assembled in vitro implies that some unifying structural principle is likely to be operative, how is the apparent morphological uniformity of these IF to be reconciled with their variations in subunit composition and size? This is the issue addressed in the present communication.

Previously, we have analyzed vimentin, desmin, and bovine epidermal keratin IF by means of scanning transmission electron microscopy (STEM) (14, 15) of unstained specimens, a technique that facilitates direct determinations of the spatial distributions of mass in macromolecules (16-18). IF of all three types were found to have average linear densities of about 37 kdalton/nm, and all samples also contained minor components at 24-26 kdalton/nm, inferred to be immature IF or some stable form of assembly intermediate. These results were consistent with the concept that vimentin, desmin, and bovine epidermal keratin IF are structurally homologous, as the subunit masses in each case are sufficiently close (53-55 kdalton) to be effectively indistinguishable. In the experiments reported here, we have extended this approach to three types of keratin IF assembled in vitro from mixtures of purified subunits with substantially different average molecular weights in order to determine any systematic dependence of IF density upon the mass of their constituent subunits.

MATERIALS AND METHODS

Purification of IF Proteins

Newborn mouse epidermal keratin subunits K1 (Mr ~67,000) and K2 (Mr ~59,000) were extracted from "4-h unattached cells" and purified by DEAE-cellulose chromatography as described (18). Human epidermis was obtained either from newborn foreskins or from adult cadaver trunk skin after separation from dermis by heating at 60°C for 40s. After extraction in urea buffer, these keratins were fractionated by DEAE-cellulose chromatography (19). The major keratins eluted by 50 mM KCl had apparent molecular weights of 65,000, 57,000, and 50,000 with a weight-average of 56,000. Those eluted by 150 mM KCl had apparent molecular weights of 52,000, 50,000, and 46,000, with a weight-average of 50,000. These combinations are respectively referred to as "heavy" and "light" subunit fractions. They, as well as the mouse keratin subunits, were separately reassembled into IF in vitro as described (11). The subunit compositions of these preparations were indistinguishable from those of the respective starting materials.

Electron Microscopy

SCANNING TRANSMISSION ELECTRON MICROSCOPY: STEM studies were carried out at the Brookhaven National Laboratory Biotechnology Resource (20). Preparation of unstained specimens by freeze-drying at constant pressure was carried out as described (21, 14, 15). The 40 keV electron probe was focused to an effective diameter of 0.25 nm, and parallel darkfield images of 512 × 512 pixels were recorded simultaneously in the large-angle and small-angle detectors. Magnifications were such that the image fields covered (2.08 μm)2 or (1.04 μm)2 of specimen area. Electron doses were ~300 electrons/nm2. The liquid-N2 cold stage maintained a specimen temperature of −150°C in these experiments.

CONVENTIONAL TRANSMISSION ELECTRON MICROSCOPY: This was performed to evaluate the efficiency of in vitro assembly experiments, using a Philips EM400T transmission microscope operating at 80 keV. A liquid-nitrogen cold-trap was in routine use. Negative staining was performed using 1% uranyl acetate.

Analysis of Data

Mass measurements were effected from the digital micrographs, using the signal from the large-angle dark-field detector only, by means of the PIC (22) and BNL (23) systems of computer programs. Statistical analyses of the resulting data, computer graphics, and other mathematical procedures were carried out with the MLAB program (24) running on a DEC-10 computer.

RESULTS

Representative STEM images of unstained keratin IF of all three types are shown in Fig. 1. Such data formed the basis of the many measurements of mass-per-unit-length compiled in Figure 2. We observed both types of human keratin IF to have a significantly higher incidence of evident internal discontinuities in density (Fig. 1) than the mouse keratin IF or indeed than any of other types previously examined (14, 15). Nevertheless, many stretches of filament could be found that were locally homogeneous by the criterion of visual inspection, and the mass measurements were carried out on such pieces. As a more quantitative test of the reliability of this criterion of homogeneity, several calculations were performed in which the digital images representing designated IF segments were integrated laterally. The resulting one-dimensional curves plot mass as a function of distance along the segments and are useful for quantitative identification of discontinuities in density. In all such cases, these quantitative results bore out the conclusion of homogeneity initially based on visual appraisal, whereas such curves also clearly showed up discontinuities where they occur (data not shown).

The distributions of density measurements for both sets of

FIGURE 1 Dark-field STEM images of unstained keratin IF reassembled in vitro from (a) human epidermal keratin subunits, (Mr) = 50,000; (b) human epidermal keratin subunits, (Mr) = 56,000; (c) mouse epidermal keratin subunits, (Mr) = 63,500. The relatively electron-dense (i.e., white) particles are tobacco mosaic virus particles, used as internal reference standards. The gray scale conveys 15 equal increments (of 0.25 kdalton/nm2) to calibrate quantitatively the representation of these images. The second darkest tone represents the average density of the carbon film substrate. × 90,000.
human keratin data (Fig. 2, a and b) contain a single major peak, skewed asymmetrically to the high-density side by an additional shoulder. Both distributions are adequately described as linear combinations of two Gaussian curves (Fig. 2, d and e), each of which represents the sampling of a single density species spread by experimental error. The distribution of mouse keratin IF data (Fig. 2c), on the other hand, forms a single symmetrical peak, slightly broader than the components present in the human keratin data. The most striking feature of these results is that the respective locations of the three principal peaks, viz. 22, 25, and 29 kdalton/nm, are significantly different in the three cases. To a very close approximation, these densities are proportional to their average subunit masses.

The same trend is observed in the respective densities of the more massive IF which are also present in minor amounts in the human keratin specimens (Table I). These two-component distributions are reminiscent of the corresponding analyses of vimentin, vimentin/desmin, and bovine epidermal keratin IF (14, 15), except that in these latter systems it was the higher density species (Class II) that formed the major components. From the observation that the lower density was most commonly encountered with short IF, or on end-segments of long IF, this type (Class I) was inferred to represent an immature "minimal form" of polymer (15). The data presented in Fig. 2 all derive from internal segments of long IF, but measurements made on end-segments or on short IF of human epidermal keratin yield, on average, the same linear densities (i.e., Class I). The values obtained are 22.0 ± 3.4 (SD) kdalton/nm (n = 19) for end-segment measurements for IF assembled from the light subunit fraction, and 24.7 ± 3.9 (SD) kdalton/nm (n = 29) for the heavy subunit fraction.

![Figure 2](https://example.com/fig2.png)

**Figure 2** Histogrammed distributions of many measurements of mass-per-unit-length performed using digital STEM images of unstained keratin filaments: (a) human epidermal keratin, light subunits (n = 189); (b) human epidermal keratin, heavy subunits (n = 289); (c) mouse epidermal keratin (n = 128). d, e, and f show the corresponding fits to these data of model distributions (continuous curves), described as sums of individual Gaussian components (dashed curves). The arrows indicate the locations of the Gaussian means, viz., the average masses-per-unit-length to which these discreet components correspond (cf. 15 and Table I).

| Keratin IF    | Class I   | Class II    | Peak width | No. of measurements |
|--------------|-----------|-------------|------------|--------------------|
| Human "light"| 21.6 ± 0.1 (83%) | 33.5 ± 0.7 (17%) | 3.8 ± 0.1 | 189               |
| Human "heavy"| 24.9 ± 0.2 (78%) | 35.1 ± 0.2 (22%) | 3.4 ± 0.1 | 289               |
| Mouse        | 29.5 ± 0.2 (100%) | —           | 4.4 ± 0.2 | 128               |

Parameters of model distributions fitted to histograms of experimental measurements of average linear density measurements effected for these three types of keratin IF. Each model distribution is a sum of Gaussian components (15). The quoted uncertainties are the standard errors estimated by the curve-fitting program (24).

TABLE I

Parameters of Model Distributions for Three Keratin IF
DISCUSSION

Masses-per-Unit-Length of Keratin IF Scale According to Average Subunit Mass

We have addressed the fundamental question of how different combinations of keratin subunits with widely varying molecular weights all form IF with apparently uniform morphologies, by using the STEM to analyze three types of such IF. To within a close approximation, the average mass-per-unit-length of these IF is proportional to the average monomer mass of the subunits contained. In other words, a fixed length of filament contains the same number of subunits in all three cases. The two human keratin IF (but not the mouse keratin) also contain minor components with average linear densities of 33 and 35 kdalton/nm, respectively, which probably correspond to the mature IF structure (15). Although based on fewer measurements and thus subject to somewhat greater uncertainty, these values are also consistent with a scaling relationship between filament density and average subunit mass.

Status of Experimental Uncertainties

The standard deviations of the Gaussian curves into which the experimental histograms were decomposed are of the order of 3–4 kdalton/nm (Table I), which is characteristic of the experimental uncertainty implicit in any single measurement. Cumulatively, however, these data define the locations of the Gaussian means with considerably higher precision. Standard error estimates for these parameters afforded by the curve-fitting procedure are 0.2–0.4 kdalton/nm for the major components and 0.5–1.0 kdalton/nm for the statistically noisier minor components. These figures are in close agreement with corresponding standard error of the mean values calculated from the standard deviations. By these criteria, the observed differences in average linear density are highly significant. As a further statistical index of whether the three sets of measurements are indeed significantly different, they were compared pairwise according to the Kolmogorov-Smirnov distribution-free test (25). In all cases, the calculated probability that the data sets represented independent samplings of the same underlying distribution was found to be negligible ($P < 10^{-4}$).

The masses of the keratin subunits under consideration were estimated by SDS PAGE, a procedure which can introduce experimental errors as high as 10–15% (19). However, the molecular weight calculated from the primary sequence of the “59 kdalton” subunit of mouse epidermal keratin, agrees to within 1% with the value previously obtained by SDS PAGE (26). Hitherto this is the only fully sequenced keratin subunit, but a similarly close correspondence holds for desmin (27). It is therefore reasonable to expect that SDS PAGE molecular weight estimates for other IF subunits are likely to be comparably reliable.

From these considerations, we conclude that the observed scaling of linear densities of IF according to the average masses of their constituent subunits has a sound statistical basis.

Comparison with IF of Other Subclasses

Other IF already characterized by STEM technology (14, 15) have closely similar subunit masses. Their linear densities are likewise similar and thus suggestive of a mutual structural homology that extends to the keratins studied here in terms of the scaling relationship noted above (cf. Table II). However, as currently defined, the latter in vitro assembly systems produce mainly (or exclusively) the minimal Class I IF structure, putative precursor to the mature Class II polymer. It is noteworthy that the relative linear densities of these two forms are close to the integer ratio of 2:3, a finding that must have significance for the as yet poorly understood higher levels of subunit organization within IF. Intriguingly, this 2:1 ratio between the density of Class I IF and the additional density of Class II IF matches the 2:1 stoichiometry between different IF subunits observed in earlier experiments on in vitro copolymerization reactions (11, 28).

A Unifying Structural Hypothesis

The simplest way to rationalize the observed scaling of filament density according to subunit mass is to postulate that all these IF are composed of building blocks that possess both conserved and variable elements. The conserved elements should have similar sizes and bonding properties, and their packing defines the overall IF geometry. The variable elements would account for the remaining mass of individual subunits. This mechanism affords a straightforward explanation for the observed phenomenon that an extensive range of keratins and other subunits assemble into morphologically similar IF that conform to the scaling relationship detected in these experiments.

Related structural concepts have been proposed earlier (29–31), primarily on the basis of protein chemical studies that...
detected similar domainal compositions for several IF subunits. These concepts have now been substantiated by recent amino acid sequence data which include the complete sequences for avian desmin (27) and a mouse 95 kdalton keratin (26), major parts for human 50 and 56 kdalton keratin (31, 32) and for hamster vimentin (33), and more fragmentary data for wool keratins (34, 35). These studies have revealed in each case a domain that is conserved in size and in the essential features of its amino acid sequence. Moreover it has the potential to form coiled-coil \( \alpha \)-helices, albeit punctuated by several non-\( \alpha \)-helical interruptions whose locations within the domain are likewise conserved (Table III). In contrast, the sizes and sequences of the non-\( \alpha \)-helical terminal domains are highly variable.

Limited chymotryptic proteolysis of mouse keratin IF has shown that their glycine-rich terminal domains can be excised by several non-\( \alpha \)-helical interruptions whose locations within the domain are conserved (Table III). In contrast, the sizes and sequences of the non-\( \alpha \)-helical terminal domains are highly variable.

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REFERENCES

1. Fraser, R. D. B., and T. P. MacRae. 1980. Molecular structure and mechanical properties of keratins. In The Mechanical Properties of Biological Materials. J. F. V. Vincent and J. D. Currey, editors. Soc. Exp. Biol. Sym. 34, Soc. Exp. Biol., London, 211-240.
2. Steinert, P. M., and J. Cantieri. 1983. Epidermal keratins. In Biochemistry and Physiology of the Skin. L. A. Goldsmith, editor. Oxford University Press, New York, 135-169.
3. Steinert, P. M., R. Zackroff, M. Aynardi-Whitman, and R. D. Goldman. 1982. Isolation and characterization of intermediate filaments. In Methods and Perspectives in Cell Biology. L. Wilson, editor. Academic Press, New York, 2A:399-419.
4. Anderson, B. H. 1981. Intermediate filaments: a family of homologous structures. J. Muscle Res. Cell Mot. 2:141-166.
5. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. Nature (Lond.). 283:249-256.
6. Steinert, P. M. 1982. Intermediate filaments. In Electron Microscopy of Proteins. J. R. Harms, editor. Academic Press, Inc., London. 1:125-166.
7. Henderson, D., and K. Weber. 1982. Electron microscopy of the cytoskeleton. In Electron Microscopy in Biology. J. D. Griffith, editor. Wiley & Sons, New York, 2:124-160.
8. Fraser, R. D. B., and T. P. MacRae. 1973. The structure of \( \alpha \)-keratin. Polymer. 14:61-67.
9. Fraser, R. D. B., T. P. MacRae, and E. Suzuki. 1976. Structure of the \( \alpha \)-keratin fibril. J. Mol. Biol. 105:547-567.
10. Day, W. A., and D. S. Gilbert. 1972. X-ray diffraction pattern of asolectin. Biochim. Biophys. Acta. 285:503-506.
11. Steinert, P. M., W. W. Idler, and S. B. Zimmerman. 1976. Self-assembly of bovine epidermal keratin filaments in vitro. J. Mol. Biol. 105:547-567.
12. Steinert, P. M., S. B. Zimmerman, J. M. Starger, and R. D. Goldman. 1978. Tensin-filaments of hamster BHK-21 cells and epidermal keratin filaments have similar structures. Proc. Natl. Acad. Sci. USA. 75:6098-6103.
13. Renner, W. W., W. Franke, E. Schmid, N. Geisler, K. Weber, and E. Mandelkow. 1981. Reconstitution of intermediate-sized filaments from denatured monomeric vimentin. J. Mol. Biol. 149:285-306.
14. Steinert, A. C., J. Wall, H. Hainfeld, and P. M. Steinert. 1982. Structure of fibrillar intermediate filaments: analysis by scanning transmission electron microscopy. Proc. Natl. Acad. Sci. USA. 79:3101-3105.
15. Steinert, A. C., J. F. Hainfeld, B. L. Trus, J. Wall, and P. M. Steinert. 1983. The distribution of mass in heteropolymer intermediate filaments assembled in vitro. J. Biol. Chem. 258:8323-8329.
16. Engel, A. 1978. Molecular weight determination by scanning transmission electron microscopy. Ultramicroscopy. 2:73-81.
17. Lammvik, M. K. 1978. Muscle thick filament mass measured by electron scattering. J. Mol. Biol. 122:55-68.
18. Steinert, P. M., W. W. Idler, M. G. Pörser, Y. Katoh, G. A. Stoner, and S. H. Yuspa. 1979. The subunit composition of the mouse epidermal keratin filament. Biochem. Biophys. Acta. 576:11-21.
19. Steinert, P. M., W. W. Idler, and W. W. Idler. 1975. The polypeptide composition of bovine epidermal \( \alpha \)-keratin. Biochem. J. 151:603-614.
20. Wall, J. 1979. Mass measurements with the electron microscope. In Introduction to Analytical Electron Microscopy. J. J. Henn, J. L. Goldstein, and D. C. Hay, editors. Plenum Publishing Corp., New York, 333-342.
21. Monson, M. W., J. Hainfeld, R. H. Haschemeyer, and J. Wall. 1981. Identification and mass analysis of human fibrinogen molecules and their domains by scanning transmission electron microscopy, J. Mol. Biol. 153:695-718.
22. Trus, B. L., and A. C. Steven. 1981. Digital image processing of electron micrographs—the PIC system. Ultramicroscopy. 6:383-386.
23. Hainfeld, J. F., J. S. Wall, and E. J. Desmond. 1982. A small computer system for micrograph analysis. Ultramicroscopy. 8:263-270.
24. Knott, G. D. 1979. MBL—a mathematical modelling tool. Comp. Programs, Biomed. 20:71-280.
25. Lindger, R. W. 1984. Statistical Theory. MacMillan, New York, 329-332.
26. Steinert, P. M., R. H. Rice, D. R. Roop, B. L. Trus, and A. C. Steven. 1983. Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. Nature (Lond.). 302:794-800.
27. Geisler, N., and K. Weber. 1982. The amino acid sequence of chicken muscle desmin (26).
provides a common structural model for the intermediate filament proteins. EMBO (Eur. Mol. Biol. Organ.) J. 1:1049–1056.
28. Steinert, P. M., W. W. Idler, F. Cabral, M. M. Gottesman, and R. D. Goldman. 1981. In vitro assembly of homopolymer and copolymer filaments from intermediate filament subunits of muscle and fibroblastic cells. Proc. Natl. Acad. Sci. USA 78:3692–3696.
29. Steinert, P. M., W. Idler, and R. D. Goldman. 1980. Intermediate filaments of baby hamster kidney (BHK-21) cells and bovine epidermal keratinocytes have similar ultrastructures and subunit domain structures. Proc. Natl. Acad. Sci. USA 77:4534–4538.
30. Geisler, N., E. Kaufmann, and K. Weber. 1982. Protein chemical characterization of three structurally distinct domains along the protofilament unit of desmin 10 nm filaments. Cell 30:277–286.
31. Hanukoglu, I., and E. Fuchs. 1982. The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins. Cell 31:243–252.
32. Hanukoglu, I., and E. Fuchs. 1983. The cDNA sequence of a Type II cytoskeletal keratin reveals constant and variable structural domains among keratins. Cell. 33:915–924.
33. Quax-Jeuken, Y. E. F. M., W. J. Quax, and Bloemendal, H. 1983. Primary and secondary structure of hamster vimentin predicted from the nucleotide sequence. Proc. Natl. Acad. Sci. USA 80:3548–3552.
34. Sparrow, L. G., and A. S. Inglis. 1980. In Proceedings of 6th International Wool Textile Research Conference, Pretoria. 2:237–246.
35. Creuziger, W. G., L. M. Dowling, and A. S. Inglis. 1980. Amino-acid sequence data from a microfibrillar protein of α-keratin. In Proceedings of 6th International Wool Textile Research Conference, Pretoria. 2:79–91.