Differential Extraction of Keratin Subunits and Filaments from Normal Human Epidermis

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Abstract. We have investigated keratin interactions in vivo by sequentially extracting water-insoluble proteins from normal human epidermis with increasing concentrations of urea (2, 4, 6, and 9.5 M) and examining each extract by one- and two-dimensional gel electrophoresis, immunoblot analysis using monoclonal antikeratin antibodies, and EM. The viable layers of normal human epidermis contain keratins K1, K2, K5, K10/11, K14, and K15, which are sequentially expressed during the course of epidermal differentiation. Only keratins K5, K14, and K15, which are synthesized by epidermal basal cells, were solubilized in 2 M urea. Extraction of keratins K1, K2, and K10/11, which are expressed only in differentiating suprabasal cells, required 4–6 M urea. Negative staining of the 2-M urea extract revealed predominantly keratin filament subunits, whereas abundant intermediate-sized filaments were observed in the 4-urea and 6-M urea extracts. These results indicate that in normal human epidermis, keratins K5, K14, and K15 are more soluble than the differentiation-specific keratins K1, K2, and K10/11. This finding suggests that native keratin filaments of different polypeptide composition have differing properties, despite their similar morphology. Furthermore, the observation of stable filaments in 4 and 6 M urea suggests that epidermal keratins K1, K2, and K10/11, which ultimately form the bulk of the protective, nonviable stratum corneum, may comprise filaments that are unusually resistant to denaturation.

KERATINS are a family of water-insoluble proteins (mol wt 40–67 kD) that form the intermediate filaments of almost all epithelial cells (9, 42). Although over 20 different human keratin polypeptides have been identified in various epithelia, hair and nails, all keratins can be classified as members of either an acidic subfamily (type I) or a relatively basic subfamily (type II) (6, 15, 19, 21, 27, 30, 37, 41, 46). At least one keratin from each subfamily is expressed in all epithelial cells, and a member from each subfamily appears to be required for filament assembly both in vivo and in vitro (8, 17, 18, 26, 29, 39).

Specific acidic and basic keratin polypeptide combinations are generally coexpressed in stoichiometric amounts, and different "keratin pairs" have been correlated with distinctive phenotypic features of epithelial differentiation (see reference 43). Thus, keratins of simple epithelia differ from those found in stratified epithelia, and differentiating suprabasal cells in various stratified epithelia (e.g., esophagus, cornea, epidermis) synthesize keratin pairs characteristic of their type of terminal differentiation. These findings have led to the hypothesis that different keratin pairs may copolymerize to form filaments which perform specific functions in maintaining the differentiated state.

Several experiments, however, have cast doubt on the significance of keratin pair specificity in filament structure and assembly. In vitro filament reassembly experiments have shown that keratins within a subfamily can be interchanged so that any Type I keratin can polymerize with any Type II keratin to form morphologically similar 10-nm filaments (18). Furthermore, injection of exogenous keratin mRNA into cultured epithelial cells, or transfection of foreign keratin cDNA, leads to synthesis and integration of "illegitimate" keratins into the native filament network, with no morphological alteration of the recipient cell (1, 13, 17). Thus, the role of keratin polypeptide diversity in filament assembly, structure, and function, remains obscure.

Normal human epidermis contains four major keratins (molecular masses 67, 58, 56.5, and 50 kD, corresponding to Moll catalogue keratins K1, K5, K10/11, and K14, respectively), as well as lesser amounts of a 65-kD keratin (K2) and another 50-kD keratin (K15) (6, 30). Several investigators have shown that the major keratin polypeptides are sequentially expressed during the course of epidermal differentiation, as cells progress from the germinative basal layer through the spinous and granular layers, to the outer cornified layer (2, 14, 38, 47). Keratin K5 (type II) and keratin K14 (type I) are expressed in basal cells, whereas keratins K1 and K10/11 (types II and I, respectively) are synthesized in suprabasal cells. The keratin pair K5 and K14 are expressed not only in epidermal basal cells, but in basal cells of almost all stratified squamous epithelia, suggesting that these keratins may provide a foundation for epithelial

stratification without imparting a particular program of differentiation (31). In contrast, expression of keratins K1 and K10/11 is restricted to epidermis and other epithelia undergoing "skin-type differentiation", suggesting that this suprabasal pair may play a role in formation of the protective, nonliving stratum corneum (30, 43, 46). In keeping with these concepts, studies of epidermal keratin polypeptide interactions in vitro have demonstrated that filaments reconstituted from keratins K5 and K14 form disperse filaments, as would be required in living (and dividing) basal cells, whereas reconstituted filaments containing keratins K1 and K10/11 have a propensity to aggregate, as occurs in formation of the stratum corneum (8). The interactions among keratin polypeptides during normal epidermal differentiation in vivo, however, are unknown.

To investigate keratin polypeptide interactions in native epidermal intermediate filaments, water-insoluble proteins from normal human epidermis were sequentially extracted with increasing concentrations of urea. We found that some of keratins K5, K14, and K15 were solubilized in 2 M urea in the form of keratin filament subunits. In contrast, extraction of keratins K1, K2, and K10/11 required concentrations of 4 M urea or greater, and these keratins were extracted as 10-nm filaments. These results suggest that keratin filaments or subunits comprised of keratins K5, K14, and K15, as found in normal human epidermal basal cells, are more soluble than filaments containing keratins K1, K2, and K10/11, as expressed in suprabasal epidermal cells. This is consistent with the idea that keratin filaments in basal cells, which include the mitotic cells of the epidermis, must be more flexible and dynamic than filaments within suprabasal cells, which must ultimately form the tough, resistant stratum corneum. In addition, these data suggest that, whereas keratin polymerization may be promiscuous among denatured keratin polypeptides in vitro, or in cultured cells manipulated to accept abnormal keratin combinations, conditions in vivo may regulate keratin interactions to form filaments with unique, differentiation-related properties.

Materials and Methods

Extraction of Epidermal Proteins

Normal human skin (typically 400–500 cm^2) was obtained from reduction mammoplasties or abdominoplasties and frozen flat at −70°C. Within 18 h, the epidermis was scraped from the frozen tissue and homogenized in ice-cold 25 mM Tris/HCl, pH 7.5, containing 1.5 M KCl, 0.5% Triton X-100, and a protease inhibitor cocktail (5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 5 μg/ml pepstatin and 10 μg/ml antipain). The homogenate was centrifuged at 15,000 *g* at 4°C for 15 min, the supernate was removed, and the pellet was washed twice with cold 25 mM Tris/HCl, pH 7.5, containing the protease inhibitor cocktail. No keratin was detected in any of these aqueous supernates, and they were routinely discarded.

The water-insoluble pellet was subsequently divided. Approximately 10% of the pellet was resuspended in 25 mM Tris/HCl, pH 8.0 containing 9.5 M urea, extracted at room temperature for 3 h, and centrifuged at 15,000 *g* for 30 min. This is a standard procedure to extract total keratins from the viable layers of the epidermis (6, 47). The supernate was referred to as the 9.5 M urea direct extract. The remainder of the water-insoluble pellet was sequentially extracted in 25 mM Tris/HCl, pH 8.0, containing increasing concentrations of urea. The pellet was first resuspended in Tris buffer containing 2 M urea, incubated for 30 min with intermittent shaking, and centrifuged at 15,000 *g* for 20 min. The supernate, containing the 2 M urea extract, was removed, and the pellet was subsequently reextracted in Tris buffer containing 4 M urea, then 6 M urea, and finally 9.5 M urea, in a similar manner. The volume of each sequential extract (as distinguished from the 9.5-M urea direct extract) was ~1 ml per 75 cm^2 surface area of the initial epidermis. The 2-M, 4-M, and 6-M urea sequential extractions included the same protease inhibitor cocktail as the aqueous extractions, and were performed at 4°C to prevent proteolysis. The 9.5-M urea extractions (both direct and sequential) were performed at room temperature.

The direct and sequential urea extractions were also performed using phosphate buffer, pH 8.0, instead of Tris, with the same results.

Gel Electrophoresis and Immunoblotting

The supernates from the four sequential urea extractions (2-, 4-, 6-, and 9.5-M urea sequential extracts) and the 9.5-M urea direct extract were analyzed on SDS gels, as previously described (6, 24). Two-dimensional gels, using nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension, were performed as described by O'Farrell et al. (32).

Individual keratins, and filaggrin and filaggrin precursors, were identified by immunoblot analysis, as previously described (6, 45). Monoclonal anti-keratin antibodies AE1 and AE3 were generously provided by Dr. T.-T. Sun (New York University). Polyclonal guinea pig anti-keratin antibody and monoclonal anti-human filaggrin antibody were purchased from ICN Immunobiologicals (Lisle, IL) and Biomedical Technologies Inc., (Stoughton, MA), respectively.

Electron Microscopy

Supernates of the 2-, 4-, and 6-M urea sequential extractions were applied to glow-discharged, carbon-coated microscope grids within 60 min of extraction (except where noted) and negatively stained with 0.75% uranyl formate, pH 4.25, as previously described (8).

For reconstitution of keratin filaments in vitro, 2-M urea sequential extracts and 9.5-M urea direct extracts were first adjusted to 9.5 M urea, 50 mM DTT and then preincubated at 37°C for 2 h, except where otherwise noted. The samples were then dialyzed 5–16 h against either 5 mM Tris/HCl, pH 7.5, 1 mM DTT or 25 mM Tris/HCl, pH 7.5 (in vitro keratin filament reassembly conditions), and negatively stained as above.

Specimens were examined in a Zeiss EM10C electron microscope operated at an acceleration voltage of 80 kV.

Results

Identification of Water-insoluble Epidermal Proteins Extracted with Increasing Concentrations of Urea

Water-insoluble epidermal proteins were sequentially extracted with increasing concentrations of urea (2 M → 4 M → 6 M → 9.5 M urea) and the sequential extracts, as well as water-insoluble proteins solubilized by direct extraction with 9.5 M urea, were analyzed on SDS gels. Coomassie blue staining showed that the 9.5 M urea direct extract contained predominantly keratins of the viable layers of normal human epidermis (keratins K1, K2, K5, K10/11, and K14 + K15, molecular mass 67, 65, 58, 56.5, and 50 kD, respectively; Fig. 1, lane *5*), as previously reported (2, 6, 30). When 1% of each sequential extract was applied, bands in the 50–67 kD "keratin molecular weight range" were visible in all four extracts (Fig. 1, lanes *1–4*). The 2 M urea extract contained relatively small amounts of numerous proteins, so that faint polypeptide bands were distributed over a wide molecular weight range (Fig. 1, lane *1*). In the 4 M urea extract, keratin bands were more pronounced (Fig. 1, lane *2*). The majority of keratin, however, was extracted with 6 and 9.5 M urea (Fig. 1, lanes *3* and *4*). Although two additional high molecular weight bands (~120 and 175 kD) were noticeable in the 6- and 9.5-M urea sequential extracts, the keratin bands were clearly dominant.

To better identify the polypeptides extracted at each urea concentration, unstained gels of the extracts were transferred to nitrocellulose for immunoblot analysis. The amount of

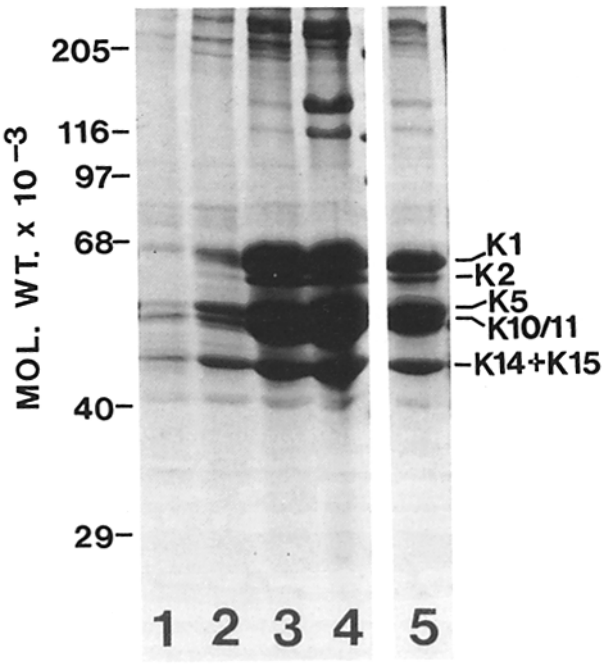


Figure 1. SDS-gel electrophoresis of water-insoluble epidermal proteins sequentially extracted with increasing concentrations of urea, stained with Coomassie blue. 1% (vol/vol) of proteins sequentially extracted with 25 mM Tris/HCl, pH 8.0, containing 2 M urea (lane *1*), 4 M urea (lane *2*), 6 M urea (lane *3*), and 9.5 M urea (lane *4*) were analyzed on 12.5% polyacrylamide gels and compared with proteins extracted directly in 25 mM Tris/HCl, pH 8.0, containing 9.5 M urea (lane *5*). Numbers on left indicate positions of molecular mass standards. On the right, keratins of the living layers of normal human epidermis are identified according to the Moll catalogue (30).

protein of each extract applied to the gel was standardized to give approximately equal intensity of the 50-kD band, as judged by fast green staining of total proteins transferred to the nitrocellulose (Fig. 2 *a*). Close inspection of the fast green-stained electroblot revealed that the 2-M urea extract contained several bands in the 50–70-kD molecular mass range that did not correspond precisely with the keratin bands in the 6- and 9.5-M urea extracts (Fig. 2 *a*; compare lane *1* with lanes *3–5*). The 2-M urea extract contained a band >67 kD and several bands in the 55–58-kD region, but bands corresponding to keratins K1, K5, or K10/11 could not be distinguished.

Keratins were identified by reacting the electroblots with monoclonal anti-keratin antibodies AE3 and AE1. In the 9.5-M urea direct extract, AE3 antibody recognizes keratins K1, K2, and K5, and AE1 antibody recognizes keratins K10/11 and K14 + K15 (Fig. 2 *b*, lane *5* and Fig. 2 *c*, lane *5*, respectively), so that together these two antibodies recognize all major epidermal keratins (6). Immunoblot analysis of the sequential extracts showed that the 2-M urea extract contained only keratin K5 (as recognized by AE3, Fig. 2 *b*, lane *1*) and keratins K14 and/or K15 (as recognized by AE1, Fig. 2 *c*, lane *1*). In the 4-, 6-, and 9.5-M urea sequential extracts, increasing amounts of keratins K1 and K10/11 were detected by the antibodies (Fig. 2 *b*, lanes *2–4* and Fig. 2 *c*, lanes *2–4*).

Immunoblot analysis using a polyclonal anti-keratin antibody agreed with the mAb staining results, suggesting that the differences in keratin composition of the sequential extracts were not due to posttranslational modifications of specific epitopes (data not shown). When the water-insoluble pellet was repeatedly extracted with 2 M urea, but no higher urea concentrations, some additional keratin K5 and keratin(s) K14 and/or K15 were solubilized, but keratins K1, K2, and K10/11 were not detected in the extract (data not shown).

During the course of epidermal differentiation, the keratinocyte cell envelope becomes increasingly insoluble. To investigate the possibility that preferential extraction of keratins K5, K14, and K15, which are the only keratins in epidermal basal cells, was due to differences in basal cell vs. suprabasal cell permeability, immunoblot analysis of the sequential urea extracts was also performed using an anti-filaggrin mAb. Filaggrin and its precursor, profilaggrin, are expressed only in the granular and cornified layers of the

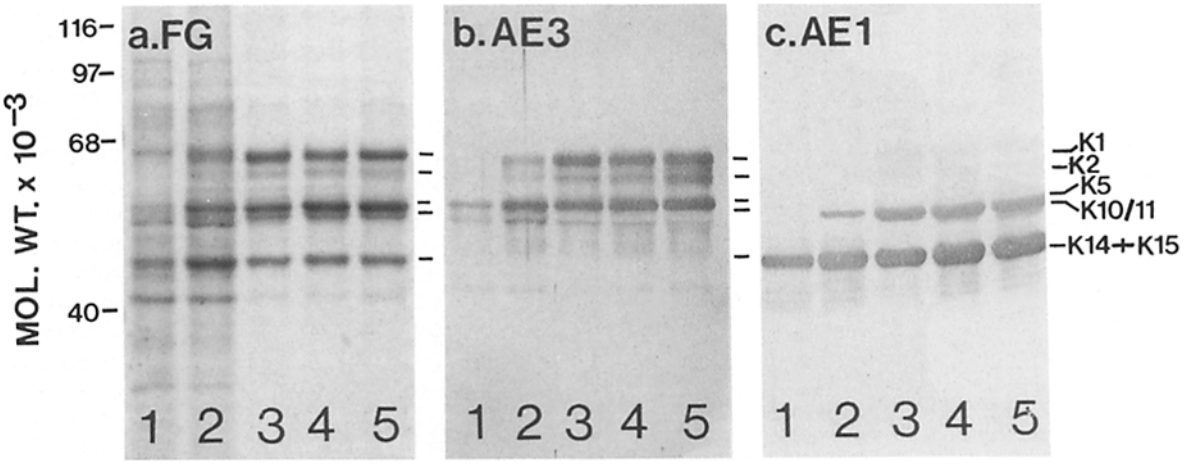


Figure 2. Immunoblot analysis of keratins sequentially extracted from normal human epidermis with increasing urea concentrations. Volumes of sequential urea extracts shown in Fig. 1 were adjusted to yield approximately equal intensity of the 50-kD band. Proteins were separated on SDS gels, transferred to nitrocellulose paper, and stained with fast green to visualize total proteins (*a*, *FG*). Lanes *1–5* designated as in Fig. 1. Parallel electroblots were subsequently reacted with monoclonal anti-keratin antibody AE3 (*b*), which recognizes keratins K1, K2, and K5, and antibody AE1 (*c*), which recognizes keratins K10/11, K14, and K15 (6). Position of molecular mass standards are indicated on left. Keratins of the living layers of human epidermis are identified according to the Moll catalogue on the right (30).

epidermis (28). Immunoblot analysis of the sequential urea extracts showed that both filaggrin and profilaggrin were extracted almost entirely with 2 and 4 M urea (Fig. 3). Thus, the difference in keratin polypeptide solubility could not be explained by decreased accessibility to the urea in differentiating suprabasal cells.

Together, these data suggested that keratins K1, K2, and K10/11 were more resistant to solubilization in 2 M urea than keratins K5, K14, and K15.

EM of Water-insoluble Epidermal Proteins Extracted with Increasing Concentrations of Urea

The sequential urea extracts were examined by EM. Negative staining of 2 M urea extracts revealed abundant heterogeneous structures ~4–10 nm wide and 50–150 nm long, as well as scattered, relatively pleomorphic structures (Fig. 4). The rod-shaped structures were similar in appearance to protofibrillar subunits formed in vitro by denatured keratins dialyzed against 2–3 M urea at neutral pH, or dialyzed against 5 mM Tris/HCl, pH 9.0–9.5 (7). The majority of subunits in the 2-M urea extract were much larger than protofilament structures (2–3 nm diameter), which are thought to represent the fundamental heterotetrameric subunit of keratin filaments (34, 35). Intermediate-sized filaments were rarely observed in the 2-M urea extract.

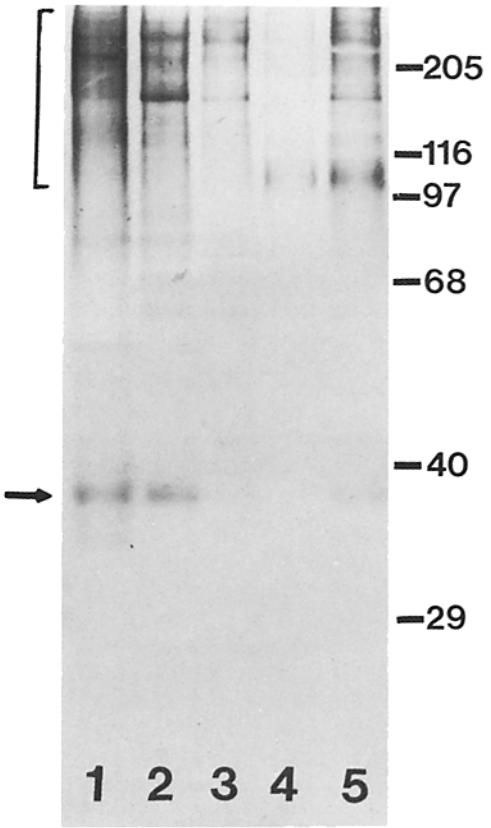


Figure 3. Immunoblot analysis of filaggrin and filaggrin precursors sequentially extracted from normal human epidermis with increasing urea concentrations. Electroblot of urea extracts as shown in Fig. 2 *a* was reacted with a monoclonal anti-human filaggrin antibody. Lanes *1–5* designated as in Fig. 1. Bracket on left denotes filaggrin precursors. Arrow on left denotes filaggrin (molecular mass 38 kD). Numbers on right indicate position of molecular mass standards ($\times 10^{-3}$).

Negative staining of the 4- urea and 6-M urea sequential extracts yielded strikingly different results. In addition to protofilamentous and protofibrillar structures, numerous webs of 10-nm diameter filaments were observed in the 4-M urea extract (Fig. 5 *a*). Some of the filaments were partially unraveled or frayed. Filaments were even more prominent in the 6-M urea extract (Fig. 5 *b*). The filaments were stable for several hours at 4°C. The urea extracted filaments appeared somewhat less compact than filaments reconstituted from denatured epidermal keratins (Fig. 5 *c*), but this may have been due to differences in the preparations (i.e., presence of urea) rather than differences in filament structure. These results were in sharp contrast to the behavior of in vitro reconstituted epidermal keratin filaments, or reconstituted desmin filaments, which rapidly disassemble into subunits when exposed to concentrations of 2 M urea or greater (7, 40).

Although keratins solubilized in 2 M urea were extracted as protofibrillar structures, they were competent to form filaments (Fig. 6 *a*). Dialyzing the extract directly against 25 mM Tris/HCl, pH 7.5, or against 5 mM Tris/HCl, pH 7.5, 1 mM DTT, yielded sparse filaments, although many were unusually long. Concentration of the extract to a final keratin concentration >0.1 mg/ml, and preincubation in 9.5 M urea, 50 mM DTT at 37°C before dialysis against filament reassembly buffer, enhanced filament formation. Even after preincubation, however, filaments formed from the 2 M urea extract were, on average, thinner than filaments reconstituted from the 9.5-M urea direct extract, and they were frequently decorated with conspicuous globular material at irregular intervals (compare Fig. 6, *a* with *b*).

Two-dimensional Gel Electrophoresis of Epidermal Proteins Preferentially Solubilized in 2 M Urea

The finding that keratins solubilized in 2 M urea were extracted as subunits, whereas keratins extracted under more severe denaturing conditions were extracted as filaments, suggested that keratins in the 2-M urea extract might be biochemically modified. In particular, previous investigation of intermediate filament dynamics during cell division and migration has suggested that phosphorylation may influence filament stability (4, 33, 44).

To determine whether keratins extracted in 2 M urea were preferentially phosphorylated, keratins in the 2-M urea extract and 9.5-M urea direct extract were resolved by two-dimensional gel electrophoresis and immunoblot analysis. Fast green staining of total proteins transferred to the nitrocellulose blots showed that the 9.5-M urea direct extract contained multiple isoelectric variants of all epidermal keratins, except a single spot for keratin K15 (Fig. 7 *a*), as previously published (6). The 2 M urea extract contained spots corresponding to keratins K5, K14, and K15, plus a single 56.5-kD spot slightly more acidic than keratin K10/11, and three distinct 49–51-kD spots between keratins K14 and K15 (Fig. 7 *b*). Immunoblot analysis using antibodies AE3 and AE1 was used to confirm the identity of the keratins. In the 9.5-M urea direct extract, AE3 antibody recognized multiple isoelectric variants of keratins K1, K2, and K5 (Fig. 7 *c*), and AE1 antibody recognized all variants of keratins K10/11, K14, and

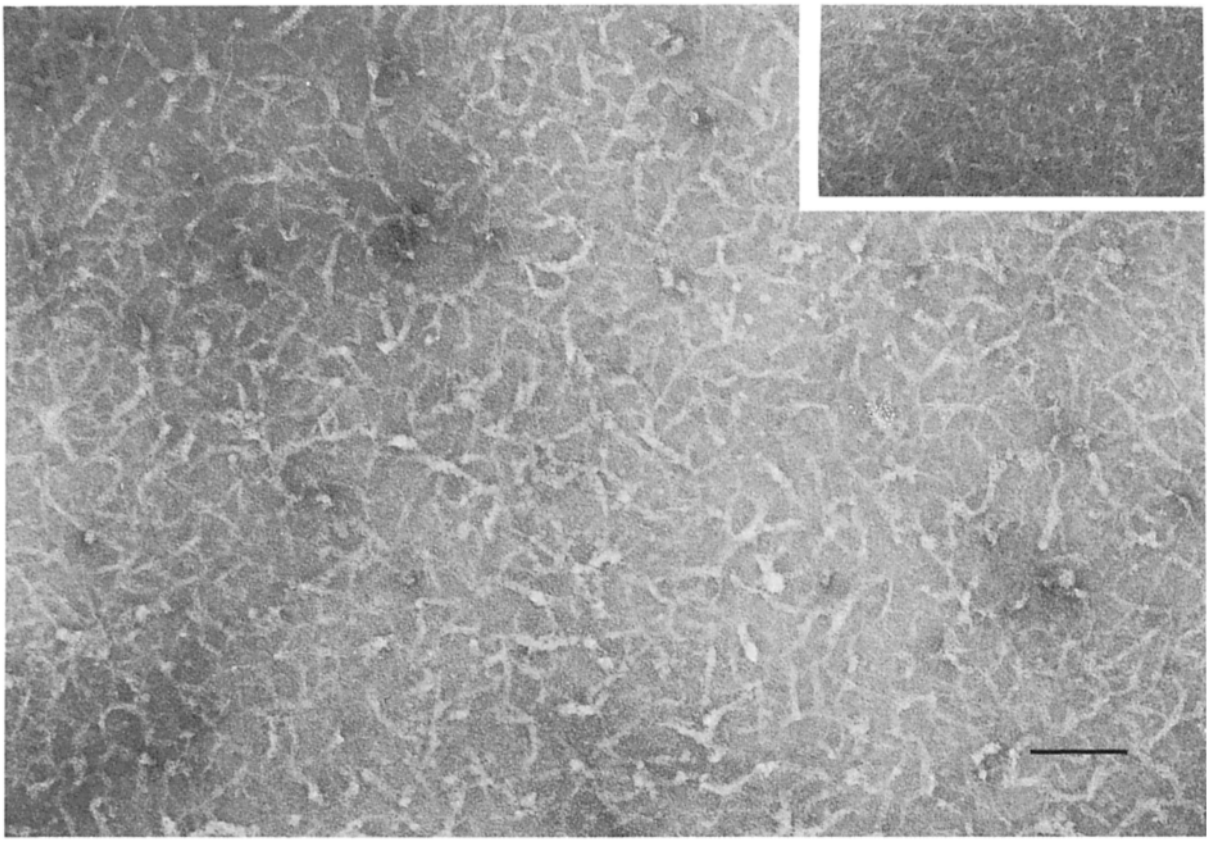


Figure 4. EM of negatively stained protofibrillar structures extracted with 2 M urea. For comparison, inset shows 2–3 nm diam protofilaments formed by epidermal keratins extracted directly in 9.5 M urea and dialyzed against 4 M urea. Bar, 100 nm.

K15 (Fig. 7 *e*), so that all apparent isoelectric variants of epidermal keratins were recognized by one of the two antibodies. In the 2-M urea extract, AE3 antibody detected predominantly the most basic (presumably unphosphorylated) variant of keratin K5, and lesser amounts of more acidic keratin K5 variants (Fig. 7 *d*). AE1 antibody recognized two isoelectric variants of keratin K14 and a single keratin K15 spot in the 2-M urea extract, as detected in the 9.5-M urea direct extract (Fig. 7 *f*). The relative amount of keratin K15 staining, however, was greater in the 2-M urea extract than in the 9.5-M urea direct extract. Although antibody staining is not quantitative, inspection of the fast green–stained electroblots also indicated that the 2-M urea extract was enriched in keratin K15 relative to the 9.5-M urea direct extract (compare Fig. 7, *a* and *b*).

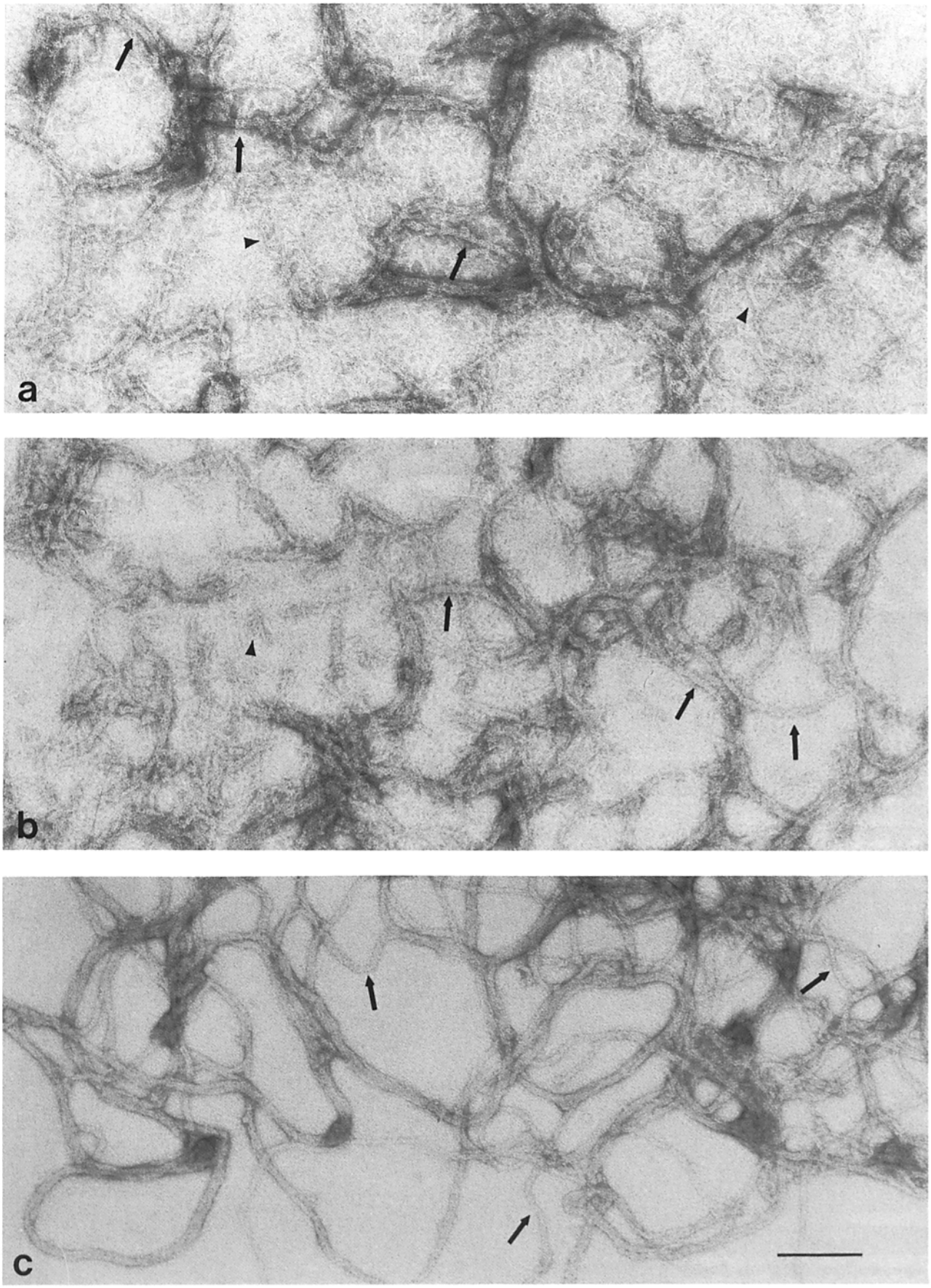
The 56.5-kD spot and the three 49–51-kD spots in the 2-M urea extract, which were visible by fast green staining, were not recognized by AE1 antibody, or other anti-keratin antibodies. The "diagonal series" of the three spots, however, is similar to the characteristic pattern of intermediate filament proteins and their proteolytic breakdown products (11, 36).

Discussion

Preferential Solubility of Keratins K5, K14, and K15 from Normal Human Epidermis

Our results demonstrate that keratins K5, K14, and K15 are more soluble than other keratins in normal human epidermis. Because K5, K14, and K15 are the only keratins expressed in epidermal basal cells (2, 14, 47), it is tempting to speculate that keratins solubilized in 2 M urea were primarily of basal cell origin. Potentially, keratins in the basal layer may be more open to denaturation, whereas association with keratins K1 and K10/11, or other intermediate filament–associated proteins in the suprabasal layers, may provide protection from denaturing agents. This idea is in keeping with early electron microscopic observations of basal cell filaments vs. spinous and granular cell filaments (3). Discrete keratin filaments were discerned in basal cells, whereas filaments appeared fused into compact, opaque fibrils in suprabasal cells. The notion that suprabasal epidermal keratins or intermediate filament–associated proteins may shield basal cell keratins is also consistent with the observed masking of antigenic sites on keratins K5 and K14 in suprabasal layers, as detected by immunohistochemical staining of frozen skin sections. Although not all keratins K5, K14, and K15 were extracted with 2 M urea, the unextracted subset could be those keratins that persist in suprabasal cells. Our experiments do not rule out the possibility that some keratins K5, K14, and K15 are simply more easily extracted from filaments containing all epidermal keratins. Filaments isolated after the 2-M urea extraction, however, showed no morphological change to support this alternative.

Intermediate-sized filaments were rarely observed in the 2-M urea extract. Heterogeneous 4–10-nm diameter struc-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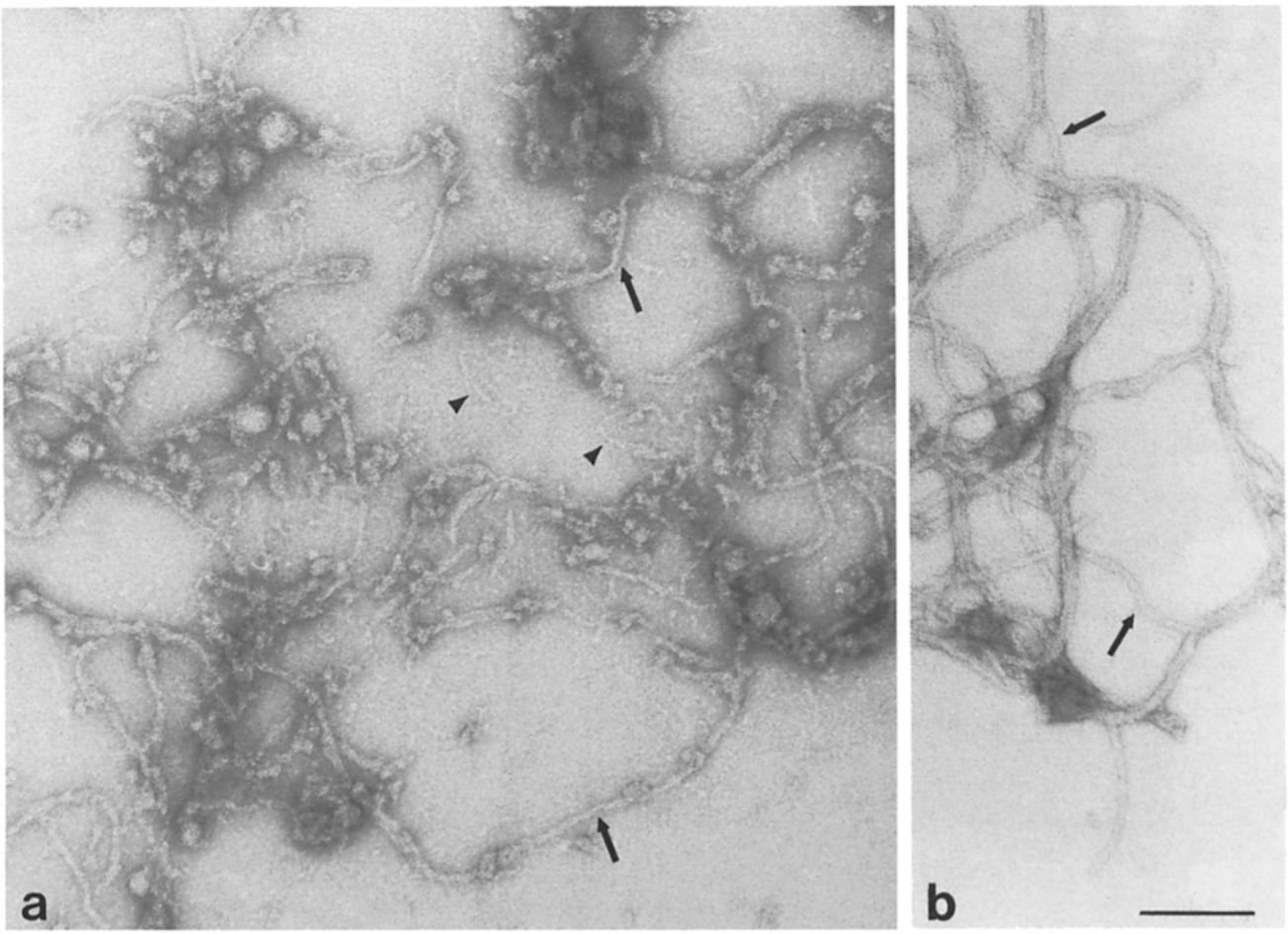

Figure 6. EM of negatively stained keratin filaments reassembled in vitro from epidermal proteins extracted with 2 M urea (*a*) and 9.5 M urea directly (*b*). Arrows and arrowheads indicate examples of individual filaments and protofibrillar subunits, respectively. Bar, 100 nm.

tures, 50–150 nm long, appeared to represent the majority of keratin extracted. Subunits of similar size have been observed as intermediates during in vitro reconstitution of filaments from denatured keratins derived from cultured human epidermal cells (7) and bovine epidermis (10). The fundamental building block of keratin filaments is believed to be a 2–3-nm-diam protofilament that is a heterotetramer (i.e., two acidic type I keratins plus two basic type II keratins) of two coiled-coil dimers (7, 34, 35). It has been suggested that two protofilaments associate laterally to form 4.5-nm protofibrils, and two to four protofibrils subsequently aggregate to form 8–12-nm filament subunits (7), which then anneal longitudinally to form full-length filaments. Accordingly, the majority of structures observed in the 2-M urea extract most probably represent aggregates, one to four protofibrils in diameter and one to four subunits long.

Keratins extracted in 2 M urea may have existed as filaments in vivo that rapidly dissociated in vitro (~1 h between exposure to 2 M urea and negative staining), or, alternatively, they may have been free subunits in the living cells. Dialysis of the 2-M urea extract against 25 mM Tris/HCl yielded filaments, indicating that keratins in the extract were competent to form filaments, and that the polypeptide concentration was high enough to maintain filaments under nondenaturing conditions. Several studies on cultured cells and some carcinomas have shown dynamic alterations in keratin filament organization during mitosis, cell movement, and treatment with specific drugs (5, 11, 16, 20, 22, 25, 44). In some cases, keratin filaments appeared to unravel into subunits. Since the basal layer contains the germinative cells of the epidermis, the 2-M urea extract could represent (or include) keratin subunits from cells undergoing mitosis. In keeping with this notion, two-dimensional gel electrophoresis demonstrated a greater proportion of keratin K15 in the

Figure 5. EM of negatively stained intermediate-sized filaments in 4 M urea (*a*) and 6 M urea (*b*) sequential extracts of water-insoluble epidermal proteins. For comparison, keratin filaments were reconstituted in vitro from epidermal keratins extracted directly in 9.5 M urea and dialyzed against 5 mM Tris, 1 mM DTT (*c*). Arrows indicate examples of individual filaments. Arrowheads denote examples of protofibrillar subunits. Bar, 100 nm.

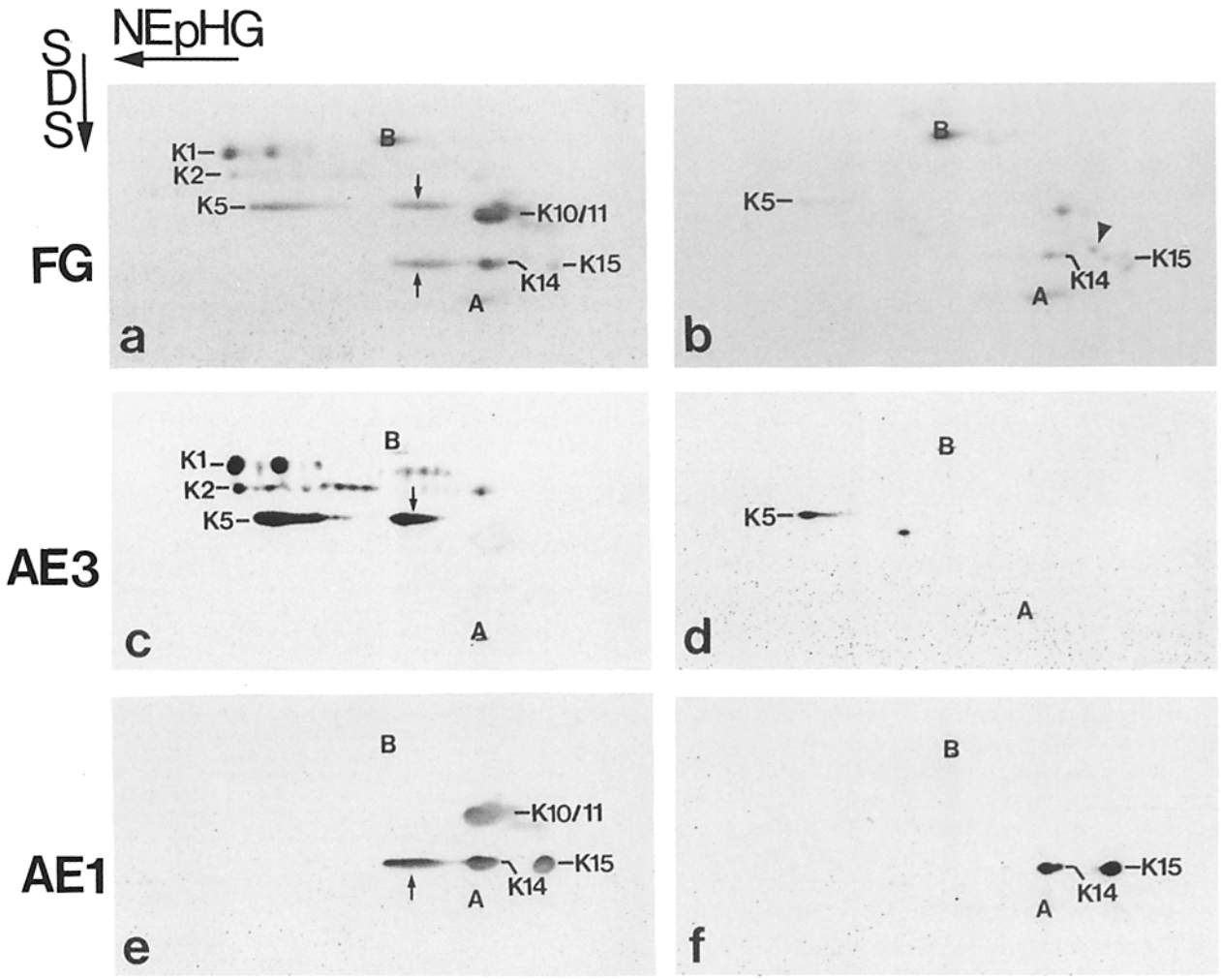

Figure 7. Two-dimensional gel electrophoresis and immunoblot analysis of epidermal keratins extracted in 9.5 M urea directly and 2 M urea. Water-insoluble epidermal proteins were extracted with either 9.5 M urea (*a*, *c*, and *e*) or 2 M urea (*b*, *d*, and *f*). Actin (*A*) and BSA (*B*) were added to each sample as internal standards. All samples were saturated with urea before separating by nonequilibrium pH gradient (NEPHGE) electrophoresis in the first dimension, and SDS gel electrophoresis in the second dimension (6, 32). Gels were electroblotted to nitrocellulose paper and stained with fast green (*FG*) to visualize total proteins (*a* and *b*). Electroblots were subsequently reacted with monoclonal anti-keratin antibody AE3 (*c* and *d*), which recognizes all basic type II epidermal keratins, and monoclonal anti-keratin antibody AE1 (*e* and *f*), which recognizes all acidic type I epidermal keratins (6). Keratins are identified according to the Moll catalogue (30). Arrows in *a*, *c*, and *e* denote complexes of keratins K5 and K14 that are not separated in the first dimension. Arrowhead in *b* denotes largest of three spots of ~50 kD that do not react with antibody AE1, but display the "staircase" pattern typical of intermediate filament proteolysis (11, 36).

2-M urea extract than in the 9.5-M urea direct extract of total epidermal keratins. Recently, increased synthesis of a 50-kD keratin, similar in charge to keratin K15, was reported in cultured squamous cell carcinoma cells induced to rapidly proliferate by treatment with retinoids (23). The increase in expression of this keratin coincided with an increase in the number of proliferating cells and a decrease in nonproliferating cells, suggesting that the 50-kD keratin may be synthesized by proliferating cells. Taken together, it is tempting to speculate that keratin K15 may be a significant component in proliferating epidermal basal cells.

Extraction of Epidermal Keratin Filaments in 4 and 6 M Urea

After extracting epidermal components soluble in 2 M urea, intermediate-sized filaments were isolated from epidermis using 4 and 6 M urea. This finding was unexpected because filaments were rarely observed in the 2-M urea extract. In addition, only protofilaments and small subunit structures have been reported in 4-M urea extracts of other epithelia and cultured epithelial cells (12, 34).

The durability of epidermal filaments, even under relatively severe denaturing conditions, could be specified by the keratins themselves or could be due to some intermediate filament-associated component(s). Recovery of filaments in the extracts was coincident with extraction of keratins K1 and K10/11. More keratins K1 and K10/11 were extracted in the 6-M urea extract than in the 4-M urea extract, and, correspondingly, more filaments were observed in the 6-M urea extract despite seemingly harsher conditions. Because filaggrin and profilaggrin (the most well-defined epidermal kera-

tin filament-associated proteins; 28) were removed in the 2- and 4-M urea extracts, they cannot be responsible for the observed filament stability. However, several other proteins were extracted in 6 M urea that might participate in maintaining filament integrity under these conditions (see Fig. 1, lane *3*). Given the unusual insolubility of desmosomes and the epidermal cell envelope, epidermal keratin filaments may also be stabilized by associations with these or other structural components of the differentiating keratinocyte.

Alternatively, extraction of filaments in 4 and 6 M urea could be due to removal of a filament dissociating factor within the 2 M urea extract. Previously, investigators have speculated on the existence of a soluble factor responsible for filament unraveling during mitosis in some cultured epithelial cells (11, 44). Extraction of such an "unraveling factor" with 2 M urea could explain why keratin filaments were recovered in the 4- and 6-M urea extracts, but not the 2-M urea extract.

Structure and Assembly of Native vs. Reconstituted Epidermal Keratin Filaments

Although native epidermal keratin filaments were stable in 4–6 M urea, reconstituted epidermal filaments disassemble into protofilament subunits under these conditions. This suggests that the organization of native filaments may differ from the organization of filaments reassembled from denatured epidermal proteins, despite their similar morphology and potentially identical composition. The instability of reconstituted filaments may result from "illicit pair" formation during in vitro reassembly. Denaturing keratin filaments in 8–9.5 M urea breaks the majority of interactions between coexpressed Type I/Type II keratin pairs. As the urea is dialyzed out and these uncoupled keratins repolymerize into filaments, they may pair with unnatural partners. In vitro studies of keratin interactions in different concentrations of urea have shown that keratin polypeptides frequently have no greater affinity for their coexpressed partner than for several other keratins (18). Illicit pairs may further polymerize into morphologically "normal" 10-nm filaments, but these structures may not be equivalent to native filaments on either the biochemical or functional level.

Although keratin promiscuity is acceptable in vitro and in cultured cells forced to express foreign keratins (13, 17, 18), specific temporal and/or spatial restrictions may be imposed in vivo. The present results are consistent with the following pathway of epidermal keratin filament assembly. Keratins K5, K14, and K15 are coexpressed and form filaments in basal cells. Acidic type I keratins K14 and K15 would be obligate partners with the basic type II keratin K5 in forming protofilaments. Basal cell filaments may be more dynamic than suprabasal cell filaments, possibly unraveling during mitosis or when basal cells detach from the basement membrane and move toward the surface. Unraveling of basal cell filaments, however, may not disrupt the fundamental protofilament subunit, thereby insuring the continued fidelity of the coexpressed keratin pair. Subsequent synthesis of keratins K1 and K10/11 in suprabasal cells would still leave only one partner available, enforcing faithful copolymerization of keratins K1 and K10/11 into protofilament subunits.

Subunits containing different keratin pairs may further segregate into separate filaments in suprabasal cells, or may integrate into the same filament network. Although our present results cannot distinguish between these possibilities, our data demonstrate that different epidermal keratins have differing physical properties. Furthermore, these data support the concept that keratin heterogeneity in differentiating epithelia provides specific functions. In the case of epidermis, basal cell keratins may be more dynamic and flexible, whereas suprabasal filaments, destined to form the rugged superficial stratum corneum, may be particularly resistant to denaturation.

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