Pairwise Assembly Determines the Intrinsic Potential for Self-Organization and Mechanical Properties of Keratin Filaments

Soichiro Yamada,* Denis Wirtz,*[†] and Pierre A. Coulombe[‡]

*Department of Chemical Engineering and [†]Department of Materials Science and Engineering, The Johns Hopkins University, Baltimore, Maryland 21218; and [‡]Departments of Biological Chemistry and Dermatology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Submitted July 19, 2001; Revised October 18, 2001; Accepted October 31, 2001
Monitoring Editor: Guido Guidotti

Most type I and II keratin genes are spatially and temporally regulated in a pairwise manner in epithelial tissues, where they represent the major structural proteins. Epithelia can be partitioned into simple (single-layered) and complex (multilayered) types. We compared the structural and mechanical properties of natural keratin polymers occurring in complex (K5-K14) and simple (K8-K18) epithelia. The intrinsic properties of these distantly related keratin filaments, whether dispersed or bundled in vitro, were surprisingly similar in all respects when at high polymer concentration. When type I and II assembly partners were switched to give rise to mismatched polymers (K5-K18; K8-K14), the interfilament interactions, which determine the structural and mechanical properties of keratin polymers, were significantly altered. We also show that a K5-K16 polymer exhibits lesser elasticity than K5-K14, which may help explain the inability of K16 to fully rescue the skin blistering characteristic of K14 null mice. The property of self-interaction exhibited by keratin filaments is likely to assist their function in vivo and may account for the relative paucity of cytoplasmic and keratin-specific cross-linkers. Our findings underscore the fundamental importance of pairwise polymerization and have implications for the functional significance of keratin sequence diversity.

INTRODUCTION

Keratins (40–70 kDa) are known as the major structural proteins of epithelial cells, where they occur as intermediate-sized cytoskeletal filaments in the cytoplasm. They are encoded by a large family comprising >40 functional genes in the human and other mammalian genomes, which can be subdivided into two major types, I and II, based on various criteria (Fuchs and Weber, 1994; Quinlan *et al.*, 1994). Keratin filaments are built from lateral and longitudinal interactions involving type I-II heterodimers (Fuchs and Weber, 1994). Most keratin genes are regulated in a pairwise, tissue-specific, and differentiation-specific manner, generating patterns that have been very useful to study epithelial tissues in health and disease (O'Guin *et al.*, 1990).

Conservation of sequence and regulation suggests a direct relationship between keratin polymers and epithelial cell structure and function. In complex epithelia, keratin filaments act as a mechanical scaffold enabling their constituent

cells to withstand deformation without breaking (Fuchs and Cleveland, 1998; Takahashi *et al.*, 1999). This function is crucial in surface epithelia, such as epidermis, oral mucosa, and hair (Coulombe *et al.*, 2000), and has been demonstrated as well for internal simple epithelia, including liver, trophoblast and placenta (Magin *et al.*, 1998; Hesse *et al.*, 2000; Tamai *et al.*, 2000; Ku *et al.*, 2001). In the liver, additionally, K8-K18 filaments protect hepatocytes against chemical toxicants and facilitates specific signaling events (Omary and Ku, 1997; Caulin *et al.*, 2000). Although there exist direct evidence to support the notion that individual keratin proteins are not functionally equivalent in the specific epithelial setting of skin epidermis (Hutton *et al.*, 1998; Paladini and Coulombe, 1999), the rationale for the multiplicity of keratin sequences remains an open question (Coulombe and Omary, 2002).

In this study, we compared the structural and mechanical properties of the major keratin filament polymers typical of simple and complex/soft epithelia and addressed the importance of pairwise polymerization. We selected the type II K5 and type I K14, which occur in the basal layer of most complex epithelia, and compared them to the type II K8 and type I K18, the most prevalent keratin pair in simple epithe-

DOI: 10.1091/mbc.01-10-0522.

[§] Corresponding author. E-mail address coulombe@jhmi.edu.

lia (O'Guin *et al.*, 1990). We also tested the mechanical properties of copolymers of K5 and K16, the latter being highly related to K14 at the primary structure level. Our findings underscore the crucial role of keratin protein complementarity in determining the nature and extent of filament-filament interactions and, hence, intrinsic mechanical properties.

MATERIALS AND METHODS

Protein Purification and Assembly

Recombinant human keratin proteins were purified as described by Coulombe and Fuchs (1990). Plasmids containing keratin sequences, pET-K5 and pET-K14 (Coulombe and Fuchs, 1990), pET-K8 and pET-K18 (Ku *et al.*, 1997), and pET-K16 (Paladini *et al.*, 1996), were transformed into *Escherichia coli* strain BL21(DE3) or BL21(DE3)pLysS for protein overexpression. All recombinant proteins used do not carry extraneous sequences. Purified type I and II proteins were mixed in Tris-buffered 6.5 M urea, and heterotypic complexes were recovered using Mono-Q ion-exchange chromatography (Amersham Pharmacia Biotech, Piscataway, NJ).

To polymerize keratin proteins, heterotypic complexes (1.0 or 0.2 mg/ml) were dialyzed against 1) 8 M urea, 25 mM Tris-HCl, pH 7.4, and 10 mM β -mercaptoethanol at 4°C; 2) 2 M urea for >2 h at 4°C; 3) 5 mM Tris-HCl, pH 7.4, and 5 mM β -mercaptoethanol, overnight at room temperature. This sequence is referred to as the standard assembly condition. The final assembly buffer was altered in some experiments in an effort to modulate filament-filament interactions in solution (Ma *et al.*, 2001). Polymerization efficiency was assessed by high-speed centrifugation ($125,000 \times g$ for 30 min) using an Airfuge (Beckman, Palo Alto, CA) followed by SDS-PAGE electrophoresis and Coomassie blue staining of pellet and supernatant fractions. Structural features of individual filaments were examined by negative staining (1% uranyl acetate, aqueous) and electron microscopy (Philips CM120, Eindhoven, The Netherlands). For this purpose sampling was restricted to the regions of the electron microscope grid support, where the outline of individual filaments could be clearly seen. Larger polymer structures, e.g., bundles, could be best examined using differential-interference-contrast (DIC) microscopy (Eclipse, Nikon, Tokyo, Japan; Ma *et al.*, 2001).

Rheology

The viscoelasticity of a polymer suspension subjected to an oscillatory shear deformation is characterized by an elastic modulus (G') and a viscous modulus (G''). For small deformation amplitudes, G' and G'' represent the in-phase and out-of-phase components, respectively, of the stress response normalized by the magnitude of the deformation (Ferry, 1980). The linear regime corresponds to the range of strains for which G' and G'' are independent of the deformation. The phase angle (δ) corresponds to the delay in the material response due to energy dissipation. δ is formally related to G' and G'' ($\delta = \arctan(G''/G')$) and is expressed in degrees. δ values of 0° and 90° are characteristic of elastic solids (e.g., steel) and viscous liquids (e.g., oil), respectively. Strain-induced yielding of a polymer occurs when the viscoelastic moduli decreases and which, for a solid-like material (i.e., $G' > G''$ in the linear range), coincides with the cross-over of G' and G'' ($\delta > 45^\circ$).

Rheological measurements were performed in a strain-controlled ARES 100 rheometer (Rheometrics, Piscataway, NJ) as described by Ma *et al.* (2001), except that a parallel-plate (25 mm in diameter) geometry was used in most experiments to reduce the sample volume (0.6 ml). For K8-K18 filament suspensions (1 mg/ml) in standard buffer condition, similar elastic moduli are obtained with 50-mm cone-and-plate (3.2 ± 1.0 dyn/cm²) and 25-mm parallel plate (5.4 ± 1.0 dyn/cm², mean $\pm$ SEM). To eliminate the interfacial component of the elasticity of the samples tested, 0.5 mg/ml dimyr-

istoyl phosphatidylcholine (P7331, Sigma, St. Louis, MO) dissolved in chloroform was applied to the air-water interface (Muller *et al.*, 1991).

Samples were loaded on the rheometer and phospholipids were applied to the air-liquid interface. Viscoelastic moduli were monitored by applying a small oscillatory deformation (10% amplitude) at a 1-rad/s frequency. Measurements were taken every 1 min to ensure that the material reached mechanical equilibrium (1.5–3 h). Afterward, the frequency response of the viscoelastic modulus was measured from 0.01–100 rad/s while maintaining a 10% strain amplitude (known as the “frequency sweep assay”). The strain dependence of the viscoelastic modulus was measured as the amplitude of strain increased from 1–600% at a fixed frequency of 1 rad/s (“strain sweep assay”). The dynamic viscoelastic modulus was calculated from the in-phase and out-of-phase components of the stress response to the deformation.

RESULTS

Complex and Simple Epithelial Keratins Are Mechanically Similar

The human K5-K14 and K8-K18 polymers were analyzed in vitro to compare the intrinsic structural and mechanical properties of major keratin systems that occur in complex and simple epithelial tissues. Despite substantial differences in primary structure, K5-K14 and K8-K18 each copolymerize efficiently in the same standard assembly buffer to form filaments that appear similar in width, length, and persistence length, as revealed by negative staining and electron microscopy (Figure 1, A, C, and E; see also Hatzfeld and Franke, 1985). When these filament suspensions are examined by DIC microscopy, occasional bundles are observed in both cases (Figure 1, F and G). The low frequency of these bundles indicates that individual filaments, whose dimensions are below the resolution afforded by light microscopy, are well dispersed in solution.

Rheological assessment of K5-K14 and K8-K18 filament suspensions at 1 mg/ml protein concentration reveals that they exhibit similar viscoelasticity over a wide range of deformation frequencies (Figure 1H). Both suspensions exhibit frequency-independent moduli, which are typical of highly constrained filament networks that do not relax during the deformation cycles. The magnitudes of the elastic modulus at 1 rad/s frequency are 7.4 ± 1.2 dyn/cm² for K5-K14 filaments and 5.4 ± 1.0 dyn/cm² for K8-K18 filaments (mean $\pm$ SEM). Both K5-K14 and K8-K18 suspensions are solid like as indicated by small δ values ($\delta_{K5-K14} = 4.1 \pm 0.6^\circ$ and $\delta_{K8-K18} = 5.2 \pm 1.5^\circ$ at 1 rad/s frequency; mean $\pm$ SEM). The viscoelastic moduli of K8-K18 filament suspensions exhibit a dependence on concentration ($G' \sim C^{1.5}$) as expected for semiflexible fibrous polymers (Morse, 1998; Palmer *et al.*, 1999). Concentration dependence is less marked for K5-K14 polymer suspensions, which retain greater elasticity than K8-K18 when at lower concentrations ($G' \sim C^{0.6}$). Given that the available estimates place keratin concentration in the several milligram per milliliter range in keratinocytes (Sun and Green, 1982; Ellis, 2001), all of the studies reported here were performed at 1 mg/ml. We conclude that, under conditions of high protein concentration in standard assembly buffer, the mechanical properties of K5-K14 and K8-K18 filaments are similar, a phenomenon that is consistent with their similar morphological structure and organization.

Figure 1. Structure, organization, and mechanical properties of simple and complex epithelial keratins. (A–D) Electron microscopy of keratin filaments negatively stained with aqueous 1% uranyl acetate. K5-K14 filaments assembled in buffer adjusted at pH 7.4 (A) and pH 7.0 (B) and K8-K18 filaments assembled at pH 7.4 (C) and pH 7.0 (D). The width of micrographs is 300 nm. (E) Sedimentation of K5-K14 and K8-K18 filament suspensions in assembly buffer at pH 7.4 and pH 7.0. S, supernatant; P, pellet. (F and G) DIC microscopy of K5-K14 (F) and K8-K18 (G) filament suspensions assembled at pH 7.4. Bar, 50 μ m. (H) Frequency (ω) dependence of the elastic G' (filled symbols) and viscous G'' (open symbols) moduli of K5-K14 (circles) and K8-K18 (squares) filament suspensions in standard assembly buffer at pH 7.4. (I and J) DIC microscopy of K5-K14 (I) and K8-K18 (J) filament suspensions assembled at pH 7.0. The scale is the same as in F. (K) Frequency (ω) dependence of the elastic G' (filled symbols) and viscous G'' (open symbols) moduli of K5-K14 (circles) and K8-K18 (squares) filament suspensions in standard assembly buffer at pH 7.0. The symbols are the same as in H. See MATERIALS AND METHODS for rheological definitions.

We hypothesized that self-induced filament interactions may differ in K5-K14 and K8-K18 pairs because they often exhibit a distinct organization in their respective host cell types (Coulombe *et al.*, 1989; Omary and Ku, 1997) and differ in their sensitivity to urea-induced denaturation (Franke *et al.*, 1983) and in their end-domain sequences (Quinlan *et al.*, 1994). To assess this potential, we polymerized K5-K14 and K8-K18 at 1 mg/ml in low ionic strength Tris-HCl buffer at pH 7.0 (Ma *et al.*, 2001). The resulting K5-K14 filaments are thicker (Figure 1B) than filaments obtained under standard conditions (Figure 1A), whereas K8-K18 filaments appear similar to those seen under standard condition (Figure 1, C and D). Both K5-K14 and K8-K18 filaments show extensive bundling at pH 7.0 as observed by DIC microscopy (Figure 1, I and J). By adjusting the focal plane, these bundles are often seen as three-dimensional parallel arrays that extend beyond 100 μ m in length, implying that they arise from lateral associations between 10-nm filaments. The viscoelastic moduli of K5-K14 and K8-K18 suspensions at 1 mg/ml

and pH 7.0 are similar in magnitude and frequency response (Figure 1K). The magnitude of the elastic modulus increases to 100 ± 21 dyn/cm² for K5-K14 filaments and to 70 ± 20 dyn/cm² for K8-K18 filaments (mean $\pm$ SEM), reflecting an increased resistance (>10-fold) to the deformation. These experiments indicate that the potential for pH-induced bundling is surprisingly similar for K5-K14 and K8-K18 pairs.

Mismatched Pairs Are Mechanically Different from the Natural Polymers

Keratin proteins undergo obligatory heteropolymerization. The general importance of this phenomenon is becoming increasingly appreciated among intermediate filaments (IF), although it is often facultative rather than obligatory (Herrmann and Aebi, 2000). Despite the tight pairwise regulation that characterizes many keratin genes, type I and II keratin proteins generally display a high affinity for one another and will copolymerize across pairing lines to form fibrous polymers (e.g., Hatzfeld and Franke, 1985; Hof-

Figure 2. Structure, organization, and mechanical properties of keratin polymers arising from mismatched pairs in standard assembly buffer (pH 7.4). (A) Sedimentation of K5-K14 and K8-K18 filament suspensions. S, supernatant; P, pellet. (B–E) K5-K18 (B and C) and K8-K14 (D and E) filament suspensions were observed using electron (B and D; bar, 300 nm) and DIC (C and E; bar, 50 μ m) microscopy. (F) Frequency (ω) dependence of the elastic (filled symbols) and viscous (open symbols) moduli of K5-K18 (circles) and K8-K14 (squares) filament suspensions. (G) Strain (γ) dependence of the elastic G' (filled symbols) and viscous G'' (open symbols) moduli of K5-K18 (circles) and K8-K14 (squares) filament suspensions. See MATERIALS AND METHODS for rheological definitions.

mann and Franke, 1997; Wawersik *et al.*, 1997). To assess the importance of pairwise assembly, we tested polymers arising from mismatched pairs, K5-K18 and K8-K14. Under standard conditions, the K5-K18 and K8-K14 pairs polymerize with high efficiency (Figure 2A). Electron and DIC micrographs reveal that K5-K18 filaments are wider than either K5-K14 or K8-K18 filaments (20–25 nm instead of 10–12 nm, see Figure 2B) and form bundles (Figure 2C). The K5-K18 filament suspension exhibits a gel-like texture, which is unusual under standard conditions, and shows a greater elastic modulus as tested by rheology (52 ± 15 dyn/cm² at 1 rad/s frequency, see Figure 2F). In the strain sweep assay, the K5-K18 polymer can withstand larger deformations compared with the natural polymers, and its moduli begin to decrease only when approaching the maximum strain ($\sim 200\%$) applicable by the rheometer (Figure 2G).

The mismatched K8-K14 pair, on the other hand, copolymerizes to form rather typical filaments, which are structurally similar to the K8-K18 copolymer by electron microscopy (Figure 2D). Similar to the natural polymers, bundles are only occasionally

seen in standard assembly buffer when using DIC microscopy (Figure 2E). The elastic modulus of the K8-K14 copolymer is much lower than that measured for natural polymers ($G' = 1.3 \pm 0.6$ dyn/cm² at 1 rad/s frequency, see Figure 2F). At high frequencies (>10 rad/s), K8-K14 filament suspensions become more liquid-like as the viscous modulus increases at the expense of the elastic modulus (Figure 2F). In the strain sweep assay, the elastic moduli of K8-K14 suspensions begin to decrease at 20% strain and the cross-over point ($G' = G''$) occurs at 140% strain (Figure 2G). Given that polymer morphology and assembly efficiency of the K8-K14 polymer are similar to those of either K8-K18 or K5-K14, such weak mechanical properties suggest that K8-K14 lacks non-steric contributions to elasticity.

The K5-K18 Pair Forms a Strong Gel

The polymer structure and elasticity of keratin filament suspensions are quite strongly dependent on pH (Figure 1), and the K5-K18 mismatched pair is no exception (Figure 3).

Figure 3. pH dependence of the structural and mechanical properties of the mismatched K5-K18 polymer. (A–D) Electron micrographs of negatively stained K5-K18 filaments assembled at pH 7.0 (A), pH 7.4 (B), pH 8.4 (C), and pH 9.0 (D). Sampling was restricted to regions of the electron microscope grid where individual polymers can be seen. Bar, 300 nm. (E) Frequency (ω) dependence of the elastic moduli G' of K5-K18 copolymers assembled at pH 7.0 (circles), pH 7.4 (squares), pH 8.4 (diamonds), and pH 9.0 (crosses). See MATERIALS AND METHODS for rheological definitions.

When pH is decreased from 7.4 to 7.0, the ultrastructural appearance of the thick K5-K18 fibers remains unchanged (Figure 3, A and B), and yet the elasticity measured by the rheometer increases by more than threefold, from 52 ± 15 to 176 ± 52 dyn/cm² (Figure 3E). In contrast, the thick K5-K18 fibers begin to unravel as the pH of the assembly buffer is increased. At pH 8.4 (Figure 3C), a mixture of 10-nm wide

filaments and thicker filaments is observed. At pH 9.0 (Figure 3D), most filaments show a normal thickness (~ 10 nm) and are short ($< 1 \mu\text{m}$). As the thick fibers unravel into 10-nm filaments, the elasticity progressively decreases to 3.1 dyn/cm² at pH 8.4 and then to 1.0 ± 0.7 dyn/cm² at pH 9.0 (Figure 3E). Independently of the pH conditions and the resulting polymer structure, however, the elastic modulus maintains its weak dependence on deformation frequency, and the polymers exhibit a solid-like behavior ($\delta < 10^\circ$). By comparison, the natural keratin polymers (K5-K14 and K8-K18) form very short filaments at pH 9.0 (~ 100 nm) and their mechanical properties are below the detection limit of the rheometer (< 0.5 dyn/cm²). Collectively, this evidence suggests that the unnatural K5-K18 combination can produce a structurally and mechanically “conventional” keratin polymer but that its sensitivity to buffer conditions in terms of promoting filament-filament bundling is dramatically different from that of K5-K14 and K8-K18. These findings further strengthen the correlation between keratin polymer structure and organization and the mechanical properties that result therefrom.

The K8-K14 Pair Forms a Weak Gel

Of the four pair combinations tested, K8-K14 clearly gives rise to the weakest polymer under standard assembly conditions. This is so even though it polymerizes efficiently to form typical intermediate-sized filaments (Figure 2). We find that progressively increasing the ionic strength of the buffer (0–20 mM NaCl) enhances K8-K14 filament bundling, as visualized through DIC microscopy (Figure 4, A–D) and increases the elastic modulus from 1.3 ± 0.6 dyn/cm² (0 mM NaCl) to 36 ± 2 dyn/cm² (20 mM NaCl; see Figure 4E). Elasticity shows a weak dependence on frequency and the δ values remain low (e.g., $7.2 \pm 1.5^\circ$ for 0 mM NaCl and 4.8 ± 0.4 for 20 mM NaCl) for all conditions tested. Even when in the presence of 20 mM NaCl, which produces the maximum amount of bundling (Figure 4D), the gain in elasticity remains significantly smaller than that seen for K5-K14, K8-K18, and especially K5-K18. Given that the K8-K14 polymer is structurally similar to K8-K18 and K5-K14, these findings underscore the critical role of surface determinants in controlling filament-filament interactions and bulk mechanical properties. Figure 5 summarizes the relationship between elasticity and salt concentration in the assembly buffer for the various keratin polymers tested.

Mechanical Properties of Mismatched Keratin Pairs and Protein Replacement Phenotypes

Keratin 14 null mice show extensive skin blistering and die shortly after birth (Lloyd *et al.*, 1995). Hutton *et al.* (1998) found that targeted expression of human K18 partially rescues the spontaneous, but not the mechanically-induced, skin blistering phenotype displayed by these mice. We (Paladini and Coulombe, 1999) showed that targeted expression of human K16 was significantly more successful in preventing blistering induced by mechanical trauma. As they get older, however, the K16 replacement mice develop alopecia and skin erosions in areas subjected to repeated rubbing, a phenomenon that is partly a function of the C-terminal 105 amino acids of the protein (Paladini and Coulombe, 1999). Here we performed rheological experi-

Figure 4. Salt dependence of the structural and mechanical properties of the mismatched K8-K14 polymer. (A–D) DIC microscopy of K8-K14 filament suspensions produced in standard assembly buffer supplemented with 0 mM (A), 5 mM (B), 10 mM (C), and 20 mM (D) NaCl. Bar, 50 μ m. (E) Frequency (ω) dependence of the elastic moduli G' of K8-K14 filament suspensions in standard assembly buffer supplemented with 0 mM (circles), 5 mM (squares), 10 mM (diamonds), and 20 mM (crosses) NaCl. See MATERIALS AND METHODS for rheological definitions.

ments to assess whether these replacement phenotypes could be correlated with the intrinsic mechanical properties of K5-K14, K5-K18, and K5-K16 polymers.

All three pairs assemble to form filaments with high efficiencies in standard assembly buffer (Figure 6A). Albeit shorter (see Paladini *et al.*, 1996), K5-K16 filaments (Figure 6B) are morphologically similar to K5-K14 ones (see Figure 1A) when observed by electron microscopy. As described above (e.g., Figure 2B), K5-K18 filaments are wider under these conditions and exhibit significantly greater elasticity than K5-K14 (Figure 2; see also Figure 6C). By comparison, the elasticity of K5-K16 filament suspensions is similar to

Figure 5. Comparison of the rheological properties of natural and mismatched keratin polymers as a function of ionic strength of the assembly buffer (0–20 mM NaCl). Open symbols designate the natural pairs K5-K14 (circles) and K8-K18 (squares). Filled symbols designate the mismatched pairs K5-K18 (circles) and K8-K14 (squares). Elastic moduli are measured under deformation conditions of 1 rad/s frequency and 10% strain. Error bars, SEM. See MATERIALS AND METHODS for rheological definitions. Note that all keratin proteins are insoluble at 50 mM NaCl, presumably due to the formation of incompetent assembly intermediates.

K5-K14 filaments over >4-decades of frequency in standard assembly buffer (Figure 6 C). When each keratin pair is subjected to increasing strain in standard assembly buffer, K5-K16 pair behaves identically to the K5-K14 pair when in the linear regime of deformation (<100%), but the cross-over point between the elastic and viscous moduli ($G' = G''$, corresponding to yielding) occurs at a deformation of 290% as opposed to 460% (Figure 6D). This difference is enhanced with addition of 5 mM NaCl to standard assembly buffer; K5-K14 no longer yields within the experimentally achievable strain (>600%), whereas the cross-over point for K5-K16 occurs at 550% of strain. The K5-K18 pair can better withstand the deformation applied and maintains its high elasticity even when large strains are applied under standard buffer conditions (Figure 6D).

We repeated the strain sweep assay in assembly buffer adjusted at pH 7.0, which induces filament-filament interactions. Compared with K5-K14, K5-K16 filaments undergo only a slight gain in elasticity (Figure 6E). However, and as documented above, the K5-K18 polymer exhibits much greater elasticity. The implications of these findings are discussed below.

DISCUSSION

Keratin Filaments Can Self-Organize into Bundles In Vitro

The mechanical properties of the cytoplasm are significantly influenced by the fibrous cytoskeletal networks it contains (Janmey, 1991). In vitro, however, concentrated suspensions of these polymers exhibit weaker elasticity than the intact cytoplasm of living cells (Yamada *et al.*, 2000). Filament cross-bridging can dramatically enhance the mechanical resilience of cytoskeletal networks, and not surprisingly, both

Figure 6. Comparison of the mechanical properties of K5-K14, K5-K18, and K5-K16 polymers. (A) Sedimentation assay of K5-K14, K5-K18, and K5-K16 in standard assembly buffer at pH 7.4. S, supernatant; P, pellet. (B) Electron micrograph of negatively stained K5-K16 polymers. Bar, 200 nm. See Figure 1A for K5-K14 and Figure 2A for K5-K18 polymers. (C) Frequency (ω) dependence of the elastic moduli G' of K5-K14 (circles), K5-K18 (diamonds), and K5-K16 (squares) filament suspensions in standard assembly buffer at pH 7.4. (D) Strain (γ) dependence of the elastic moduli G' of K5-K14 (circles), K5-K18 (diamonds), and K5-K16 (squares) filament suspensions in standard assembly buffer at pH 7.4. (E) Frequency strain (γ) dependence of the elastic moduli G' of K5-K14 (circles), K5-K18 (diamonds), and K5-K16 (squares) filament suspensions in assembly buffer at pH 7.0, which promotes filament cross-bridging. See MATERIALS AND METHODS for rheological definitions.

keratin IF and F-actin polymers are believed to function mostly as “organized networks” in vivo (Janmey, 1991; Coulombe *et al.*, 2000). An impressive number of ubiquitous proteins can specifically bind F-actin and organize it into various types of suprafilamentous arrays (e.g., bundles, orthogonal networks; Tseng *et al.*, 2001). Such F-actin networks in vitro display viscoelastic properties that approximate those observed in the subcortical cytoplasm of living cells (Yamada *et al.*, 2000). In striking contrast, there is no known “organizing activity” that is ubiquitous, keratin-specific, and can account for the bundled organization of keratin polymers in vivo. Instead, keratin IFs seemingly rely on a combination of intrinsic determinants and interactions with general cytoskeletal cross-linkers, many of which are concentrated at adhesion complexes (Fuchs and Karakesisoglou, 2001), for their organization and function in vivo (Coulombe *et al.*, 2000).

The ability of keratin filaments to undergo self-induced bundling has been demonstrated in studies involving polymers reconstituted in vitro from purified proteins (e.g., Eichner *et al.*, 1986; Ma *et al.*, 2001). For instance, the K1-K10 natural pair, which is characteristic of differentiating epidermal keratinocytes in vivo, readily forms large filament aggregates when polymerized in vitro (Eichner *et al.*, 1986). This corresponds largely to the natural state of the K1-K10 polymer either in differentiating keratinocytes (Coulombe *et al.*, 1989) or when these keratin proteins are ectopically expressed in the β -cells of transgenic mouse pancreas (Blessing *et al.*, 1993). Here we show that minor modifications to the standard in vitro assembly conditions, e.g., slightly lowered pH or increased ionic strength, cause K5-K14 or K8-K18 filaments to form large parallel bundles that can be visualized using DIC light microscopy. Correlating with this bundling is a sol-gel phase transition and much enhanced mechanical resilience, as shown by rheology (Table 1) and

particle tracking (Ma *et al.*, 2001). Comparable alterations in ionic strength also affect the rate of IF network assembly in vitro (Herrmann *et al.*, 1999). The measurements made in our study were taken at equilibrium, such that the observed

Table 1. Summary of keratin micromechanical properties

Pairing	Character ^a	Mechanical resilience ^b	
		Dispersed state ^c	Bundling-promoting conditions ^c
K5-K14	Solid like	Weak	Moderate-strong
K8-K18	Solid like	Weak	Moderate
K5-K18	Solid like	Moderate	Very strong
K8-K14	Solid like	Very weak	Weak
K5-K16	Solid like	Weak	Moderate

Keratin micromechanical properties are based on rheological measurements taken at 1 mg/ml protein concentration and under small deformation conditions (frequency = 1 rad/s and γ = 1–10%).

^a Sample “character” is reflected by the phase angle δ , which can range from values of 0° (pure solid) to 90° (pure liquid). For all pairings tested, δ < 10°, indicating a solid-like character. By comparison, a suspension of F-actin at 1 mg/ml has a δ of 25°.

^b Mechanical resilience is reflected by the magnitude of the elastic modulus G' . A rating of “weak” implies that $G' < 10$ dynes/cm²; “moderate” implies that $10 \text{ dynes/cm}^2 < G' < 100 \text{ dynes/cm}^2$, and “strong” implies that $100 \text{ dynes/cm}^2 < G' < 400 \text{ dynes/cm}^2$. A suspension of disperse F-actin at 1 mg/ml exhibit a G' of 5–10 dynes/cm².

^c Suspensions of disperse polymers were generated by subjected keratins to standard low ionic strength assembly conditions at pH 7.4. Bundled polymers were generated by adding 10 mM NaCl to the assembly buffer.

effects can be ascribed to enhanced filament-filament interactions rather than faster assembly. Optimal buffer conditions for keratin polymerization in vitro are of unusually low ionic strength. Attempts to make these conditions more physiological, e.g., through the addition of salt, cause keratin filaments to form bundles or aggregates; the latter may therefore correspond to the "natural state" of the polymer under physiological conditions. Our studies, summarized in Table 1, extend previous ones (e.g., Steinert *et al.*, 1976; Eichner *et al.*, 1986; Hofmann and Franke, 1997; Ma *et al.*, 2001) in showing that self-induced bundling may be an intrinsic property of most if not all keratin polymers and likely contributes to their organization and function in vivo (Ma *et al.*, 2001).

Pairwise Assembly Specifies Filament-Filament Interactions and Mechanical Properties

The findings reported here also highlight the fundamental importance of pairwise keratin assembly in determining the potential for filament-filament interactions, which in turn controls the intrinsic mechanical potential of the polymer. Two important observations were made.

First, the K5-K14 and K8-K18 polymers show a similar potential for self-organization, and exhibit similar mechanical properties across a range of polymer size and organization when tested at high polymer concentration (1 mg/ml) in vitro (Table 1). This finding is interesting in light of the profound differences exhibited by these two pairs in terms of primary structure (Quinlan *et al.*, 1994) and distribution in vivo (O'Guin *et al.*, 1990). With the limitation that it is based on in vitro studies involving pure polymers, this outcome suggests that the functional significance of keratin sequence multiplicity may not reside at the level of intrinsic polymer properties per se. Of course, significant differences may arise from the manner with which these polymers are distributed and organized within their host cell types in vivo, as well as how they are regulated by posttranslational modifications and/or other proteins (Omary and Ku, 1997). Hutton *et al.* (1998) showed that replacing K14 with K18 can only marginally rescue the skin fragility phenotype associated with a K14 null mutation in transgenic mice. On the one hand, it may be that K18, the replacement protein, was not expressed to sufficiently high levels in these mice. On the other hand, however, it may be that the "illegitimate nature" of the K5-K18 pairing contributed to this functional insufficiency. Based on the in vitro rheological findings reported here, in fact, one could predict that a complete substitution of the K5-K14 polymer with K8-K18, or vice versa, may produce a better functional outcome in this type of experimental setting. More sophisticated mouse experiments are needed to test this speculation.

Second, pairwise keratin assembly matters, in that the identity of the type I and type II keratins involved in copolymerization exerts an important influence on self-interactions and hence mechanical properties in vitro. Polymers arising from mismatched keratin pairs show either higher (K5-K18) or lower (K8-K14) resilience than the natural polymers, in all conditions tested (Table 1). The greater elasticity exhibited by K5-K18 polymer suspensions is a direct consequence of a strong tendency to form long bundles of 25- to 30-nm-wide filaments. The lower elasticity and weaker resilience observed for K8-K14 is more interesting because this

mismatched polymer is structurally similar to the natural ones tested (K5-K14 and K8-K18). This finding points directly to the significant role of nonsteric interactions between filaments in modulating the mechanical properties of keratin filament suspensions, as well as to the fundamental importance of type I-type II keratin protein complementarity. These elements are further supported by our discovery of differences in rheological properties for the K5-K14, K5-K19, and K5-K14ΔT polymers (Bousquet *et al.*, 2001; also this study) and the discovery by Hofmann and Franke (1997) that copolymers of K8 and various type I keratins exhibit different behaviors when assessed by viscometry. It shall prove very interesting to examine the properties of other keratin natural keratin pairs, including K4-K13 (oral mucosa), K3-K12 (cornea), K6-K16 (wounded tissues), and especially K1-K10 (epidermis), to see whether our findings involving K5-K14 and K8-K18 are generally applicable.

Mechanical Properties In Vitro as a Predictor of In Vivo Phenotypes

Keratins 14 and 16 are both expressed in complex epithelial tissues and represent the two most related type I keratins at the level of primary structure. The N-terminal head and central rod domains are ≈90% identical in these two proteins; they diverge significantly only at the distal end of their ≈50 residue nonhelical tail domain (Paladini *et al.*, 1996). Still, we find that alterations in either pH or ionic strength does not enhance the mechanical resilience of the K5-K16 polymer to the same degree as K5-K14 (Table 1). Based on this evidence, one would predict that the targeted expression of K16 may not fully rescue the epithelial fragility phenotype exhibited by K14 null mice. This largely corresponds to what was discovered in transgenic mice in which this complementation experiment was carried out (Paladini and Coulombe, 1999). The K16 replacement mice are initially wild-type in appearance, indicating functional redundancy. Over time, however, they develop alopecia and skin erosion in areas of repeated mechanical trauma. It is worth noting that the failure of K16 to completely rescue the mouse K14 null phenotype could be partly attributed to differences within their C-terminal 105 amino acids (Paladini and Coulombe, 1999). It is tempting to speculate that the differences seen in filament bundling and rheological properties for K5-K14 and K5-K16 are due in part to their nonhelical tail domains (Bousquet *et al.*, 2001).

As mentioned above, Hutton *et al.* (1998) found that targeted expression of human K18 could only effect a marginal rescue of skin blistering in K14 null mice. The K18 replacement mice were largely spared from the "spontaneous" blistering exhibited by 2- to 4-d-old K14 null mice, but their skin was highly susceptible to mechanical friction (Hutton *et al.*, 1998). Our rheology-based finding that the K5-K18 polymer behaves as a stronger gel than K5-K14, independently of the buffer conditions applied (Table 1), contrasts sharply with the phenotype of the K18 replacement mice. The reasons for this discrepancy are not clear (see above). A simple explanation would be that the optimal structure-function relationships displayed the K5-K14 polymer in the natural context of a basal skin keratinocyte are not duplicated by the K5-K18 polymer. It may also be that the enhanced resilience exhibited by K5-K18 occurs in vivo as it does in our hands in vitro but does not translate into a positive gain for basal skin

keratinocytes. Studies of pure keratin polymers in vitro can only offer an incomplete picture of how they are put to work in a cell. The increasing availability of biophysical methods to probe the mechanical properties of living cells (Yamada *et al.*, 2000) offers an opportunity to assess how these in vitro measurements relate to in vivo properties.

ACKNOWLEDGMENTS

These studies were supported in part by National Institutes of Health grant AR42047 to P.A.C. and National Science Foundation grants CTS9812624, CTS0072278, and DB19729358 to D.W.

REFERENCES

- Baribault, H., Penner, J., Iozzo, R.V., and Wilson-Heiner, M. (1994). Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes Dev.* 8, 2964–2973.
- Blessing, M., Ruther, U., and Franke, W.W. (1993). Ectopic synthesis of epidermal cytokeratins in pancreatic islet cells of transgenic mice interferes with cytoskeletal order and insulin production. *J. Cell Biol.* 120, 743–755.
- Bousquet, O., Ma, L., Yamada, S., Gu, C., Idei, M., Takahashi, K., Wirtz, D., and Coulombe, P.A. (2001). The non-helical tail domain of keratin 14 promotes filament bundling and enhances the mechanical properties of keratin intermediate filaments in vitro. *J. Cell Biol.* 155, 747–753.
- Caulin, C., Ware, C.F., Magin, T.M., and Oshima, R.G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J. Cell Biol.* 149, 17–22.
- Coulombe, P.A., Bousquet, O., Ma, L., Yamada, S., and Wirtz, D. (2000). The ‘ins’ and ‘outs’ of intermediate filament organization. *Trends Cell Biol.* 10, 420–428.
- Coulombe, P.A., and Fuchs, E. (1990). Elucidating the early stages of keratin filament assembly. *J. Cell Biol.* 111, 153–169.
- Coulombe, P.A., Kopan, R., and Fuchs, E. (1989). Expression of keratin K14 in the epidermis and hair follicle: insights into complex programs of differentiation. *J. Cell Biol.* 109, 2295–2312.
- Coulombe, P.A., and Omary, M.B. (2002). Hard and soft principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol.* (in press).
- Eichner, R., Sun, T.T., and Aebi, U. (1986). The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. *J. Cell Biol.* 102, 1767–1777.
- Ellis, R.J. (2001). Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr. Opin. Struct. Biol.* 11, 114–119.
- Ferry, J. (1980). *Viscoelastic Properties of Polymers*, New York: Wiley.
- Franke, W.W., Schiller, D.L., Hatzfeld, M., and Winter, S. (1983). Protein complexes of intermediate-sized filaments: melting of cyto-keratin complexes in urea reveals different polypeptide separation characteristics. *Proc Natl Acad Sci USA* 80, 7113–7117.
- Fuchs, E., and Cleveland, D.W. (1998). A structural scaffolding of intermediate filaments in health and disease. *Science* 279, 514–519.
- Fuchs, E., and Karakesisoglou, I. (2001). Bridging cytoskeletal intersections. *Genes Dev.* 15, 76–84.
- Fuchs, E., and Weber, K. (1994). Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* 63, 345–382.
- Hatzfeld, M., and Franke, W.W. (1985). Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombinations of purified polypeptides. *J. Cell Biol.* 101, 1826–1841.
- Herrmann, H., and Aebi, U. (2000). Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. *Curr. Opin. Cell Biol.* 12, 79–90.
- Herrmann, H., Haner, M., Brettel, M., Ku, N.O., and Aebi, U. (1999). Characterization of distinct early assembly units of different intermediate filament proteins. *J. Mol. Biol.* 286, 1403–1420.
- Hesse, M., Franz, T., Tamai, Y., Taketo, M.M., and Magin, T.M. (2000). Targeted deletion of keratins 18 and 19 leads to trophoblast fragility and early embryonic lethality. *EMBO J.* 19, 5060–5070.
- Hofmann, I., and Franke, W.W. (1997). Heterotypic interactions and filament assembly of type I and type II cytokeratins in vitro: viscometry and determinations of relative affinities. *Eur. J. Cell Biol.* 72, 122–132.
- Hutton, E., Paladini, R.D., Yu, Q.C., Yen, M., Coulombe, P.A., and Fuchs, E. (1998). Functional differences between keratins of stratified and simple epithelia. *J. Cell Biol.* 143, 487–499.
- Janmey, P.A. (1991). Mechanical properties of cytoskeletal polymers. *Curr. Opin. Cell Biol.* 3, 4–11.
- Ku, N.O., Gish R., Wright T.L., and M.B. Omary. (2001). Keratin 8 mutations in patients with cryptogenic liver diseases. *N. Engl. J. Med.* 334, 1580–1587.
- Ku, N.O., Wright, T.L., Terrault, N.A., Gish, R., and Omary, M.B. (1997). Mutation of human keratin 18 in association with cryptogenic cirrhosis. *J. Clin. Invest.* 99, 19–23.
- Lloyd, C., Yu, Q.C., Cheng, J., Turksen, K., Degenstein, L., Hutton, L., Fuchs, E. (1995). The basal keratin network of stratified squamous epithelia: defining the function of K15 in the absence of K14. *J. Cell Biol.* 129, 1329–1344.
- Ma, L., Yamada, S., Wirtz, D., and Coulombe, P.A. (2001). A “hot-spot” mutation alters the mechanical properties of keratin filament network. *Nat. Cell Biol.* 3, 503–506.
- Magin, T.M., Schroder, R., Leitgeb, S., Wanninger, F., Zatloukal, K., Grund, C., and Melton, D.W. (1998). Lessons from keratin 18 knockout mice: formation of novel keratin filaments, secondary loss of keratin 7 and accumulation of liver-specific keratin 8-positive aggregates. *J. Cell Biol.* 140, 1441–1451.
- Morse, D.C. (1998). Viscoelasticity of concentrated isotopic solutions of semiflexible polymers: 1. Model and stress tensor. *Macromolecules* 31, 7030–7043.
- Muller, O., Gaub, H.E., Barmann, M., and Sackmann, E. (1991). Viscoelastic moduli of sterically and chemically cross-linked actin networks in the dilute to semidilute regime: measurements by an oscillating disk rheometer. *Macromolecules* 24, 3111–3120.
- O’Guin, W.M., Schermer, A., Lynch, M., and Sun, T.-T. (1990). Differentiation-specific expression of keratin pairs. In *Cellular and Molecular Biology of Intermediate Filaments*, eds. R. D. Goldman and Steinert, P.M., New York: Plenum Publishing, 301–334.
- Omary, M.B., and Ku, N.O. (1997). Intermediate filament proteins of the liver: emerging disease association and functions. *Hepatology* 25, 1043–1048.
- Paladini, R.D., and Coulombe, P.A. (1999). The functional diversity of epidermal keratins revealed by the partial rescue of the keratin 14 null phenotype by keratin 16. *J. Cell Biol.* 146, 1185–1201.
- Paladini, R.D., Takahashi, K., Bravo, N.S., and Coulombe, P.A. (1996). Onset of re-epithelialization after skin injury correlates with a reorganization of keratin filaments in wound edge keratinocytes: defining a potential role for keratin 16. *J. Cell Biol.* 132, 381–397.

- Palmer, A., Mason, T.G., Xu, J., Kuo, S.C., and Wirtz, D. (1999). Diffusing wave spectroscopy microrheology of actin filament networks. *Biophys. J.* 76, 1063–1071.
- Quinlan, R., Hutchinson, C., and Lane, B. (1994). Intermediate filament proteins. *Protein Profile* 1, 779–911.
- Steinert, P.M., Idler, W.W., and Zimmerman, S.B. (1976). Self-assembly of bovine epidermal keratin filaments in vitro. *J. Mol. Biol.* 108, 547–567.
- Sun, T.T., Green, H., (1982). Keratin filaments of cultured human epidermal cells: formation of intermolecular disulfide bonds during terminal differentiation. *J. Biol. Chem.* 253, 2053–2060.
- Takahashi, K., Coulombe, P.A., and Miyachi, Y. (1999). Using transgenic models to study the pathogenesis of keratin-based inherited skin diseases. *J. Dermatol. Sci.* 21, 73–95.
- Tamai, Y., Ishikawa, T., Bosl, M.R., Mori, M., Nozaki, M., Baribault, H., Oshima, R.G., and Taketo, M.M. (2000). Cytokeratins 8 and 19 in the mouse placental development. *J. Cell Biol.* 151, 563–572.
- Tseng, Y., Fedorov, E., McCaffery, J.M., Almo, S.C., Wirtz, D. (2001). Micromechanics and microstructure of actin filament networks in the presence of the actin-bundling protein human fascin: a comparison with α -actinin. *J. Mol. Biol.* 310, 351–366.
- Wawersik, M., Paladini, R.D., Noensie, E., and Coulombe, P.A. (1997). A proline residue in the α -helical rod domain of type I keratin 16 destabilizes keratin heterotetramers and influences incorporation into filaments. *J. Biol. Chem.* 272, 32557–32565.
- Yamada, S., Wirtz, D., and Kuo, S.C. (2000). Mechanics of living cells measured by laser tracking microrheology. *Biophys. J.* 78, 1736–1747.