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A 'hot-spot' mutation alters the mechanical properties of keratin filament networks

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Keratins 5 and 14 polymerize to form the intermediate filament network in the progenitor basal cells of many stratified epithelia including epidermis, where it provides crucial mechanical support. Inherited mutations in K5 or K14 result in epidermolysis bullosa simplex (EBS), a skin fragility disorder. The impact that such mutations exert on the intrinsic mechanical properties of K5/K14 filaments is unknown. Here we show, by using differential interference contrast microscopy, that a 'hot-spot' mutation in K14 greatly reduces the ability of reconstituted mutant filaments to bundle under crosslinking conditions. Rheological assays measure similar small-deformation mechanical moduli. For crosslinked solutions of wild-type and mutant keratins. The mutation, however, markedly reduces the resilience of crosslinked networks against large deformations. Single-particle tracking, which probes the local organization of filament networks, shows that the mutant polymer exhibits highly heterogeneous structures compared to those of wild-type filaments. Our results indicate that the fragility of epithelial cells expressing mutant keratin may result from an impaired ability of keratin polymers to be crosslinked into a functional network.

We previously showed that suspensions of in vitro-assembled, wild-type K5/K14 filaments feature properties akin to those of visco-elastic solids when tested by rheological methods. In support of this, the elastic modulus, G', measured for a K5/K14 sample at 0.5–1.0 mg ml⁻¹ is typically 30–50 dynes cm⁻², a value that is ten times greater than the viscous modulus, G". Accordingly, the phase shift, δ, is very low, usually 8–10° (see Methods for rheological definitions). Pure intermediate filaments (IF) of keratin in suspension are still intrinsically too weak to account for their supportive functions in epithelial cells. In fact, even in vitro, it is likely that filament crosslinking makes a significant contribution to the mechanical properties of keratin IFs in vivo. In this study, we devised in vitro conditions that result in a network of crosslinked keratin filaments with enhanced mechanical resilience. We explored these conditions to assess the impact that inherited mutations exert on the intrinsic mechanical properties of keratin IFs in suspension. To this end, we studied keratin polymers containing K14G35S-C, a missense mutation discovered in the K14 gene of a large number of patients who suffer from a severe form of EBS. This arginine residue, located at the beginning of the α-helical rod domain, is highly conserved among type I keratin genes and represents a mutational hot-spot in keratinopathies.

Apart from components of cell-adhesion complexes, very few filament crosslinking proteins are IF-specific and widely distributed. To circumvent this limitation, we exploited alternative buffer conditions with the aim of enhancing filament-filament interactions in solution and thus mimicking the effect of crosslinkers in vitro. The optimal buffer conditions for epidermal keratin polymerization in vitro are of exceptionally low ionic strength (5 mM Tris–HCl and 5 mM β-mercaptoethanol, pH 7.4; ref. 9) but they promote the almost complete assembly of K5/K14 into dispersed IFs that are several μm long (Fig. 1a). Various modifications of these conditions affected a liquid–gel phase transition (see Methods) without much impact on K5/K14 filament structure. These include lowering the pH of the assembly buffer from 7.4 to 7.0 (Fig. 1b), adding a small amount of salt (for example, 10 mM NaCl), or substituting Tris–HCl for a phosphate–ion–buffered salt (data not shown). Under such conditions, gelation of the polymer sample correlated with the formation of large fibrous bundles that were seen using differential interference contrast (DIC) light microscopy (Fig. 1c). Relatively small K5/K14 bundles were only rarely detected using DIC imaging when standard buffer conditions were applied (data not shown). The results showed that, in solution, filament–filament interactions of K5/K14 polymer were modulated by minor changes in buffer conditions in the absence of other exogenous factors.

We subjected the wild-type K5/K14 polymer to rheological studies to test whether its physical properties are altered by enhanced filament crossbridging. When small deformations were applied, the magnitude of the visco-elastic moduli was similar in Tris–HCl buffer at pH 7.0 and at pH 7.4, but the phase shift value reproducibly decreased to ~5° and remained at this low level over an extended range of frequencies (Fig. 2a, b). Strikingly, the K5/K14 polymer suspension offered much higher resilience against large deformations at pH 7.0 compared with pH 7.4; that is, the linear regime is extended (Fig. 1b, c). Even at the maximal deformation imposed in the strain/weep assay, the polymer softened but did not yield (Fig. 2c; see Methods for definitions). The elastic modulus G' shows a large bump characteristic of strain-induced hardening (Fig. 2e, arrow). A similar outcome was seen when wild-type K5/K14 polymers were assembled in the presence of 10 mM NaCl or in phosphate buffer (data not shown). Rheology therefore extended the DIC microscopy and centrifugation data in showing that minor modifications to the buffer conditions can enhance interactions between assembled keratin filaments in a way that mimics polymer crossbridging.

We next assessed the impact that the K14R35S-C missense mutation (in which an arginine residue at position 125 of K14 is replaced by a cysteine) exerts on the mechanical properties of keratin filaments in suspension. When assembled under standard buffer conditions at pH 7.4, individual K5/K14R35S-C filaments were significantly shorter than wild-type filaments (data not shown) and only ~30% of the protein pool was retrieved in the pellet fraction (Fig. 1f). However, the rheological properties exhibited by the K5/K14R35S-C mutant polymer were largely similar to those of the wild-type K5/K14 polymer (Fig. 2a, b).
Figure 1. Keratin polymers. a, b, d, Electron micrographs of keratin polymers as visualized by negative staining (1% uranyl acetate) and electron microscopy. Sampling was restricted to regions of the electron microscopic grid in which individual polymers can be seen. Scale bar represents 250 nm. c, e, DIC imaging of keratin polymers under a light microscope. Scale bar represents 30 μm. Wild-type K5/K14 filament suspensions were assembled in Tris-HCl buffer at pH 7.4 (a) or pH 7.0 (b, c); K5/K14R_140,C filament suspensions were assembled in Tris-HCl buffer at pH 7.0 (d, e). f, g, Polymerization efficiency of keratin polymers (8 μg protein) by high-speed centrifugation and isoelectric focusing analysis of the pellet (p) and supernatant (s) fractions. Assembly was in Tris-HCl buffer at pH 7.4 (f) or at pH 7.0 (g).

Figure 2. Rheological assessment of bulk properties of keratin polymers. a, b, Comparison of wild-type K5/K14 (circles) and mutant K5/K14R_140,C (triangles) polymers assembled in Tris-HCl at pH 7.4 (standard condition). d, e, Comparison of wild-type K5/K14 (circles) and mutant K5/K14R_140,C (triangles) polymers assembled in Tris-HCl at pH 7.0 (conditions promoting filament crossbridging). Rheological assays were as follows: a, d, frequency-dependent elastic modulus G’ (filled symbols), and phase angle δ (open symbols) of keratin suspensions; b, e, strain dependence of elastic modulus G’ (filled symbols) and phase angle δ (open symbols) of the same keratin suspensions. Values are means ± s.d. from three independent experiments. ω, shear frequency; γ, shear amplitude. c, f, Time-resolved stress–strain relationship experiments were carried out at a 100% strain amplitude. Data corresponding to the first cycle of shear are shown; subsequent shear cycles induced a slight, clockwise rotation of the figure, which stabilized after three or four cycles of shear. Data in c were obtained at pH 7.4 and pH 7.0. Samples were as follows: wild-type K5/K14 at pH 7.4 (black circles); wild-type K5/K14 at pH 7.0 (white circles); mutant K5/K14R_140,C at pH 7.0 (triangles). Data in f were obtained in the presence of 10 mM NaCl. Samples were as follows: wild-type K5/K14 (circles); mutant K5/K14R_140,C (triangles). σ, output stress.
Figure 3 Assessment of local visco-elastic properties by particle tracking. Purified type I-type II keratin heterotypic complexes were assembled in Tris-Cl buffer at pH 7.4 or pH 7.0 in the presence of fluorescent beads and then analyzed.

a. Examples of the motion shown by single particles embedded within the wild-type (WT) K5/K14 polymer assembled at pH 7.4 or pH 7.0, and within the mutant (Mut) K5/K14R<sub>187</sub>C polymer assembled at pH 7.0.

b. Average MSD as a function of time. Values are means ± s.d. obtained from 20 independent particles for each polymer. Filled circles, K5/K14 polymers in Tris-Cl buffer at pH 7.4; open circles, K5/K14 polymers in Tris-HCl at pH 7.0; filled diamonds, K5/K14R<sub>187</sub>C polymers in Tris-Cl at pH 7.4; open diamonds, K5/K14R<sub>187</sub>C polymers in Tris-HCl at pH 7.0.

although subtle differences were apparent when these two polymers were subjected to larger deformations (data not shown). A significantly different outcome was observed when the K5/K14R<sub>187</sub>C mutant polymer was tested under conditions promoting filament crossbridging. As judged from the centrifugation assay, >95% of the K5/K14R<sub>187</sub>C polymer was retrieved in the pellet fraction after assembly at pH 7.0 (Fig. 1g). Electron microscopy indicated that the structural features (for example, length and regularity of width) of the K5/K14R<sub>187</sub>C mutant polymer may have been improved at pH 7.0 compared with pH 7.4 assembly conditions (Fig. 1d). Under DIC microscopy, however, significantly fewer filament bundles were seen in K5/K14R<sub>187</sub>C preparations compared with wild-type (a rare example is shown in Fig. 1c). A similar outcome was seen in the presence of 10 mM NaCl at pH 7.4 and in phosphate buffer (data not shown). When subjected to deformations of progressively larger amplitudes in the rheometer, the K5/K14R<sub>187</sub>C mutant polymer began to "soften" at ~10% of strain and yielded (Δ > 45°) at ~300% of strain (Fig. 2c). This was in striking contrast to wild-type K5/K14 which maintained its elasticity for much larger values of strain (Fig. 2c). At strain γ = 630%, for instance, phase angle values of δ = 71.0 ± 0.4° and δ = 30.6 ± 8.1° were measured for the mutant and wild-type polymer, respectively. Moreover, the K5/K14R<sub>187</sub>C polymer exhibited only mild strain-hardening before yielding when at pH 7.0 (Fig. 2e), or in the presence of 10 mM NaCl or in phosphate buffer (data not shown). Such differences in the behaviour of mutant and wild-type polymers were consistent between experiments (compare error bars in Fig. 2a, b, and d, e).

To analyze strain-hardening more accurately, we monitored shear stress σ(t) in a continuous manner as a fixed oscillatory deformation χ(t) (of amplitude 10–100%) applied to the polymer suspension. In this instance, strain-hardening was captured in real time through a faster-than-linear increase of σ(t) compared to χ(t) as the deformation neared the end of its cycle. We observed that the stress σ(t), which measures the resistance to deformation, increased rapidly with deformation for wild-type K5/K14 under filament-crossbridging conditions (pH 7.0; Fig. 2c). In fact, the stress increased faster than linearly at high amplitudes, a hallmark of strain-hardening. In contrast, the mutant specimen exhibited a slow increase in stress with increasing strain; that is, it exhibited little strain-hardening under the same conditions (Fig. 2e). A similar outcome was seen when testing wild-type and mutant keratin samples in the presence of 10 mM NaCl, although the K5/K14R<sub>187</sub>C polymer exhibited some strain-hardening under such conditions (Fig. 2f). Likewise, doubling the keratin protein concentration in the pre-assemble samples (from ~10 μM to ~20 μM) improved the mechanical resilience of the K5/K14R<sub>187</sub>C polymer under large deformations, but it remained significantly weaker than the wild-type (data not shown). Together, these data show that the K5/K14R<sub>187</sub>C mutant polymer, although it has properties akin to those of visco-elastic solids, has a lesser capacity to withstand large deformations, and yields rapidly under conditions in which the wild-type K5/K14 polymer shows resilience and strain-hardening.

The bulk rheological properties of wild-type K5/K14 and K5/K14R<sub>187</sub>C polymer suspensions were similar when the deformation applied was small (Fig. 2). However, the structural features of these two polymers (Fig. 1) indicate that there should be significant differences in local properties within these suspensions. To address this issue, we carried out single-particle tracking experiments in which the displacement of fluorescent particles embedded in the polymer sample was monitored as a function of time. The tracking of several independent particles (n ≥ 20) within a sample allows the computation of an average value, the mean-square displacement (MSD), the magnitude of which reflects the impact of local friction forces. The MSD value should be irrevocable with time for purely elastic materials (such as rubber), whereas its magnitude should exhibit a linear dependence upon time for purely viscous materials (such as oil). Representative examples of the brownian motion exhibited by a fluorescent bead embedded in the wild-type (at pH 7.4 and 7.0) and mutant (at pH 7.0) polymers are shown in Fig. 3a. We tracked particles embedded within the wild-type polymer solution for at least 1 min before they were lost from the plane of focus. In contrast, particles embedded in the K5/K14R<sub>187</sub>C mutant suspension could be tracked for only a few seconds before they were lost from the sampling field. These differences imply that the local environment is less constrained within the mutant polymer compared with the wild-type polymer. In support of this, the MSD values for wild-type K5/K14 showed little if any time dependence (Fig. 3b). The flat nature of the curve signifies that the wild-type polymer behaves like an elastic material. The mutant polymer, on the other hand, showed a much greater time dependence in its MSD profile, and the values calculated after 5 s were >1,000-fold larger than those for the wild-type polymer (Fig. 3b). These data reveal significant differences in the local mechanical properties between wild-type K5/K14 and K5/K14R<sub>187</sub>C. They also strengthen the correlation between the dimensions of individual polymers (most importantly length), the ability to form a crosslinked gel, and the resulting mechanical resilience.

Whether the weaker mechanical properties of the K5/K14R<sub>187</sub>C mutant keratin polymer stem from alterations in the molecular architecture or in the size of individual polymers, or both, is
unclear. The gain in elasticity afforded by adding cross-linkers to suspensions of F-actin filaments shows a direct dependence upon the length of individual polymers. The idea that EBS (and other keratin-based disorders) can be an "F network disorder" is supported by the discovery of mutations in plectin in a rare form of EBS. How our findings with K3/K4 H1.0-C are applicable to the pathogenic mechanisms of keratin-based disorders is not clear at present. The K4 H1.0-C mutation, or its equivalent in other type I keratin genes, is associated with severe forms of blistering diseases, and patients bearing these mutations show significant alterations in the organization of keratin IF networks in relevant epithelial cells. Not all instances of EBS and other keratin-based disorders are, however, associated with such alterations in keratin IF organization.

Likewise, not all keratin mutations result in obvious defects in 10-nm filament structure when assembly is tested by in vitro polymerization and electron microscopy. Methods required to test mutations that affect polymer structure in a manner that differs from the hot-spot K4 H1.0-C mutation.

Methods

Protein purification.

Plasmids pET-K3, pET-K4, and pET-K4H1.0-C were transformed into Escherichia coli strain BL21 (DE3) pLysS to generate milligram amounts of the human recombinant proteins. Proteins were purified by ion-exchange chromatography on a Hi-Trap Q column (Amersham) as described. Heterotypic complexes containing type I and type II keratins in an equimolar ratio were prepared and purified in 6 M urea. In all cases, the purity of the proteins, as determined by SDS-PAGE, was > 99%.

Assembly conditions.

Keratin protein concentration was fixed at 0.5 mg/mL (corresponding to a 50-mM urea concentration) in all studies unless specified otherwise. Keratins were reconstituted and polymerized by serial dialysis against three buffers. The first two buffers were the same: (1) 10 mM Tris, 25 mM KCl, and 10 mM β-mercaptoethanol, pH 7.4 for 4 h; and (2) 7 M urea, 5 mM Tris- HCl and 5 mM β-mercaptoethanol, pH 7.4, for 1 h. Depending upon the experiment, the third and final buffer consisted of (3) 5 mM Tris-HCl and 5 mM β-mercaptoethanol, pH 7.4 (standard conditions), (4) 5 mM Tris-HCl and 5 mM β-mercaptoethanol, pH 7.0 (ref. 22), or (5) 5 mM Tris-HCl, 5 mM β-mercaptoethanol and 10 mM NAC, pH 7.4. Dialysis was carried out overnight at room temperature.

Polymer evaluation.

The physical state of the polymer (solid or liquid) was assessed by inverting the Eppendorf tube containing a small volume of polymer solution. If the sample retained its original shape, it was scored as a gel; otherwise, it was scored as fluid-like. Assembly efficiency was determined by subjecting the assemblies to centrifugation (15,000 g for 30 min in an Airfuge, Beckman). Supernatant and pellet fractions were analyzed by SDS-PAGE and Coomassie blue staining. Keratin assemblies were examined by negative staining (4°C, 0.5% sodium acetate); and electron microscopy before and after freeze drying. To assess assembly formation, assemblies were examined using DSC microscopy (40, 1.14 NA immersion objective, Nikon).

Rheological measurements.

The polymers (0.1 nL) to be compared were assembled in parallel on the same day. Visco-elastic measurements were obtained using a strain-controlled 30-mL cone and plate Rheometrics ARES 100 rheometer (Rheometrics Inc., Piscataway, New Jersey) as described. The linear equilibrium values of the storage and the loss modulus (G' and G"), i.e., the viscous modulus (G'') of the IF polymer suspensions were measured by setting the amplitude of the oscillatory strain at γ = 1% and sweeping from low to high frequency. Stress-dependent visco-elastic moduli were measured by subjecting the polymers to three cycles of oscillatory deformation increasing amplitude (1% to 10% of γ = 1%) and measuring the maximum magnitude of the stressed state. To assess strain hardening, stress was continuously monitored during eight cycles of oscillatory shear deformation. Rheological experiments were repeated at least three times using independent protein preparations, findings were consistent between experiments.

Rheological definitions.

The visco-elastic response of a polymer subjected to a shear deformation is characterized by an elastic (storage) modulus (G') and a viscous (loss) modulus (G''). For small deformations, G' and G'' represent the phase and out-of-plane components, respectively, of the stress induced within the polymer by the imposed deformation, normalized to the magnitude of the deformation. In the linear regime, which corresponds to the range of strains for which G' and G'' are independent of the applied deformation, the elastic modulus G' varies between 30 and 50 times cin for solid-type keratin IF suspensions. The G'' value is larger than G' by nearly one order of magnitude, and accordingly, the phase shift (δ = arctan (G'G'')) which reflects the relative values of these two moduli, is small (6 ± 1°).

Given that δ values of 6° and 90° are characteristic of elastic solids (such as steel) and viscous liquids (such as oil), respectively, such data show that suspensions of keratin IFs behave like visco-elastic solids. By definition, yielding of a polymer placed under strain occurs when the value of G' becomes smaller than G'' (δ > 60°), reflecting a transition from a solid-like to a liquid-like behavior. Softening refers to a reduction in the G''/G' ratio, and is an increase in the phase shift between the input strain and the output stress (see ref. 8).

Probing suspension properties through particle tracking.

Fused keratin proteins were mixed with 1-μm-diameter fluorescent polystyrene particles. Polynucleus, Warrington, Pennsylvania before polymerization. Particles-containing suspensions (40 μL) were placed into a square-shaped well cultured by one layer of double-stick 3D Scotch tape opposed to a glass microscope slide, and sealed with a glass coverslip (top) and insert grease (on open sides). Motions of particles undergoing brownian motion in (fluorescent) suspensions were captured using a VH-100 ST camera (I mage MI, Mariemont, City, Indiana), using a Sinon frame grabber (Scrios, Frederick, Maryland) and NIH image software (NIH, Bethesda, Maryland). Images were acquired at 15 frames per s for a period of 5-6 s. The positions of particles along the x- and y-axes of the focal plane were determined by intensity-weighted centroid calculation of particle areas. From the trajectories of particle, MSD values were calculated. See ref. 12 for further details of this type of analysis.

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