Keratin Filament Suspensions Show Unique Micromechanical Properties*

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All epithelial cells feature a prominent keratin intermediate filament (IF) network in their cytoplasm. Studies in transgenic mice and in patients with inherited epithelial fragility syndromes showed that a major function of keratin IFs is to provide mechanical support to epithelial cell sheets. Yet the micromechanical properties of keratin IFs themselves remain unknown. We used rheological methods to assess the properties of suspensions of epidermal type I and type II keratin IFs and of vimentin, a type III IF polymer. We find that both types of IFs form gels with properties akin to visco-elastic solids. With increasing deformation they display strain hardening and yield relatively rapidly. Remarkably, both types of gels recover their preshear properties upon cessation of the deformation. Repeated imposition of small deformations gives rise to a progressively stiffer gel for keratin but not vimentin IFs. The visco-elastic moduli of both gels show a weak dependence upon the frequency of the input shear stress and the concentration of the polymer, suggesting that both steric and non-steric interactions between individual polymers contribute to the observed mechanical properties. In support of this, the length of individual polymers contributes only modestly to the properties of IF gels. Collectively these properties render IFs unique among cytoskeletal polymers and have strong implications for their function in vivo.

Nearly 30 years ago, a third major cytoskeletal entity comprised of 10-nm-wide filaments was discovered in the cytoplasm of a wide variety of cell types (1). This novel cytoskeletal polymer was given the name “intermediate filaments” (IFs), a designation that reflects the notion that their diameter is intermediate between those of F-actin (6–8 nm) and microtubules (25 nm). Subsequent studies showed that a large collection of heterogeneous proteins are capable of self-polymerization into IFs. The superfamily of IF genes currently features >50 members classified into five major types (2). Type I-type IV IF proteins occur in the cytoplasm of specific cell types, whereas the type V lamin proteins are primary constituents of the nuclear lamina in all nucleated cell types. The signature common to all IF proteins is a tripartite domain structure in which nonhelical N-terminal (head) and C-terminal (tail) domains flank a 310–350-amino acid-long α-helical domain (2–4). This central domain features heptad repeats of hydrophobic residues (a, b, c, d, e, f, and g, where a and d are generally nonpolar), which play an essential role during self-polymerization (2, 4, 5).

The function of cytoplasmic IF proteins remained elusive for a number of years. Although their structural features, abundance, and intracellular organization suggested a mechanical role, the discovery of viable cell lines missing IF proteins altogether (6–8) along with the lack of obvious consequences when IF networks are disrupted in cultured cell lines (9) suggested that cytoplasmic IF proteins are not essential for housekeeping functions. Direct evidence for a mechanical role was provided by the experimental disruption of IF networks in the context of cells and tissues in vivo. Indeed, targeted expression of dominant negative mutants that profoundly alter the organization of keratin IFs in the epidermis of transgenic mice was found to cause fragility syndromes wherein the cell population affected would rupture when subjected to relatively mild mechanical trauma (10–12). Similar findings came out of subsequent studies involving the expression of mutated IF genes in mouse tissues other than skin (13). A formal confirmation of this function came from the introduction of null mutations for specific IF genes in mouse, starting with keratin ones (14–16) and followed up by others since then (17–19). Collectively, these studies established that cytoplasmic IFs are a major determinant of cellular integrity, a role that is especially important in cell types subjected to frequent mechanical trauma such as epithelial sheets, muscle, and fibroblasts (see Ref. 20 for a review).

Largely as a result of the early mouse work reported above, mutations were discovered in patients suffering from a variety of skin fragility syndromes that are characterized by a dominant inheritance, an altered IF network at the ultrastructural level, and cell lysis upon trivial mechanical trauma (20–22). The clinical manifestations of these various skin diseases are primarily determined by the identity of the keratin gene affected and the nature of the mutation altering its primary sequence (20–22). Missense mutations were also found later on in patients suffering from inherited cell fragility syndromes involving epithelia other than skin (23–26) and nonepithelial tissues such as muscle (27). Consistent with its primary role during self-assembly, the vast majority of mutations discovered in the human population so far affect amino acid residues located within the central α-helical domain (20–22). Overall, the nature of these clinical phenotypes substantiate the experimental evidence for a mechanical role by IF networks.

Conceptually, the molecular defect(s) responsible for these clinical disorders may reside within the IF backbone itself, in
the organization of the filaments in the cytoplasm, or in some aspects of their regulation that pertains to their mechanical properties. Surprisingly, however, there are relatively few studies of the mechanical properties of IFs, and there are none that address the properties of keratins. Here we report on the micromechanical properties of keratin IF assemblies, which we studied using a strain-controlled cone-and-plate rheometer.

**EXPERIMENTAL PROCEDURES**

**Protein Purification—**Plasmids pET-K5 and pET-K14 (28), pET-K14PN150 (29), and pET-Vimentin (30) were transformed into *Escherichia coli* strain BL21 (DE3) to generate milligram amounts of the corresponding recombinant human proteins (31). After solubilization in urea-containing buffer, the proteins were purified by ion exchange chromatography on a Hi-Trap Q column followed by a Mono Q column (both from Amersham Pharmacia Biotech) as described (32). Native human keratins were purified from human foreskin keratinocytes in primary culture as described (32). Protein purity was routinely assessed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining, and the material used for rheological studies was >99% pure.

**Polymerization—**Assembly experiments and rheological studies (see below) were conducted at concentrations ranging from 200 to 1500 μM in a 1.6-ml sample volume. Recombinant vimentin was polymerized by serial dilution against three buffers as described (33): (a) 8 M urea, 5 mM Tris-HCl, 5 mM β-ME, pH 8.5, at room temperature; (b) 5 mM Tris-HCl, 5 mM β-ME, pH 8.5, at 4 °C; and (c) 25 mM Tris-HCl, 5 mM β-ME, 160 mM NaCl, pH 7.5, at room temperature. Purified type I and type II keratins were mixed in a ~45:55 molar ratio at a final concentration of 500 μM in 6 mM urea buffer, incubated for 1 h, and fractionated by anion exchange chromatography on the Mono Q column. Collected fractions were analyzed by SDS-polyacrylamide gel electrophoresis and those containing type I-type II heterotetramers were pooled as described (28, 32). Assembly of recombinant and native keratin proteins was achieved by serial dialysis against the following two buffers at room temperature: (a) 9 M urea, 25 mM Tris-HCl, 10 mM β-ME, pH 7.4, and (b) 5 mM Tris-HCl, 5 mM β-ME, pH 7.4. All assemblies were examined by negative staining and electron microscopy prior to their use for rheological studies. Polymerization efficiency was determined by subjecting the final assemblies (~8–10 μg of proteins) to centrifugation at 100,000 × g for 30 min at 4 °C (Airfuge, Beckman, Palo Alto, CA). Supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining and quantitated by gel scanning densitometry (32).

**Rheology—**Quantitative viscoelastic measurements (34) were obtained using a strain-controlled 50-mm cone-and-plate Rheometrics ARES 100 rheometer (Rheometrics Inc., Piscataway, NJ). The temperature of the sample (1.5 ml) was fixed at 25 °C to within 0.1 °C, and the cone-and-plate tools were enclosed in a custom-made vapor trap to prevent buffer evaporation. The prepolymerized keratin solution was placed between the cone-and-plate tools and allowed to rest for 30 min prior to the recordings. The linear equilibrium values of the storage or elastic modulus G’ (ν) and loss or viscous modulus G” (ω) of the IF polymer suspensions were measured by setting the amplitude of the oscillatory strain at ν = 1% and sweeping from low to high frequency ω. The strain-dependent viscoelastic moduli were measured by subjecting the polymers to three cycles of oscillatory deformation of increasing amplitude at 1 rad s⁻¹; G’ and G” were computed from the maximum magnitude of the measured stress and associated phase shift (35, 36). In a separate assay we measured the time-dependent stress induced in suspension of keratin and vimentin IFs subjected to oscillatory shear deformation in real time. This last assay was repeated for increasing magnitudes of deformation. Polymer samples were routinely examined by electron microscopy and SDS-polyacrylamide gel electrophoresis at the conclusion of each rheological experiment to assess whether the size or concentration of the protein(s) had changed during the course of the experiment.

**RESULTS**

**Description of the IF Polymers Tested—**The strategy applied for the characterization of the viscoelastic properties of epidermal keratin IFs was as follows. Purified recombinant proteins were used for most experiments given the large amounts of proteins required. We chose to work with the type II keratin 5 (K5), the type I keratin 14 (K14), and a mutated version of the latter, K14PN150, in which the N-terminal 150 amino acids of K14 have been deleted. Keratins are unique among IF proteins in that the polymerization process requires the involvement of type I and type II sequences in a 1:1 molar ratio (5). We selected K5 and K14 because they are naturally co-expressed in the progenitor basal layer of stratified epithelia (37), are relatively well characterized (28), and are the main target genes in epidermolysis bullosa simplex, an inherited skin blistering disease (20–22). We tested the short filament samples produced by co-expression of K5 and K14PN150 (29) to assess the contribution of individual polymer length to the viscoelastic moduli of keratin filament suspensions. We also examined the properties of an IF suspension reconstituted from native keratins purified from human foreskin keratinocytes in primary culture. As shown previously (32), the keratin profile of such keratinocytes includes the type II K5 and K6 and the type I K14 and K17 along with small amounts of K16, another type I keratin. Finally, we assessed the behavior of vimentin, a type III IF protein (2) that can homopolymerize into filaments and whose viscoelastic properties have been characterized previously (38–40).

The IF polymers tested were obtained by subjecting (a) purified type I-type II keratin heterotetramers (in Tris-buffered urea) or (b) vimentin protofilaments (in a Tris buffer at a basic pH) to polymerization in vitro. Representative examples of the samples used in our studies are shown in Fig. 1. As reported (28), polymerization of recombinant K5 and K14 results in long and flexible filaments that, as judged by the imperfect criterion of negative staining and electron microscopy, appear to be well dispersed (Fig. 1B). Quantitative measurements showed that these filaments have, on average, a 10.5-nm diameter and a 2.9-μm length. A similar outcome is seen when polymerizing purified native human epidermal keratins under identical conditions (data not shown). In both instances, ~90–95% of the initial protein pool is found in the pellet fraction in a standard centrifugation assay, reflecting the suitability of the polymerization...
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Fig. 2. Frequency-dependent elastic (filled symbols) and loss moduli (open symbols) of 1 mg/ml suspensions of K5-K14 (circles), K5-K14PN150 (triangles), and vimentin (squares) filaments. Inset, concentration dependence of the elastic modulus for suspensions of K5-K14 filaments measured at 0.01 rad/s (circles), 0.1 rad/s (squares), and 1 rad/s (triangles). c, protein concentration.

The visco-elastic moduli of wild-type keratin and vimentin IF polymers are relatively independent of the frequency of shear over an extended range of frequencies ($10^{-2}$ rad/s $< \omega < 10^2$ rad/s; Fig. 2). Moreover, stress relaxation experiments conducted over a period of 1000 s do not display a clear terminal regime that would reflect the final relaxation of the sheared polymers (data not shown). We also find that the viscoelastic modulus displayed by keratin and vimentin IF polymers is a weak function of concentration (see data for wild-type K5-K14 IFs in Fig. 2, inset), much weaker than that observed for F-actin and predicted by classical models of nonassociating polymers (36, 43). The weak concentration dependence of the plateau modulus along with the large relaxation time are not consistent with the currently accepted models that describe the rheology of polymer solutions. Collectively, these observations suggest that the motion of individual filaments is greatly restricted within the polymer suspension and that nonsteric interactions between polymers contribute to the plateau modulus observed.

We next assessed the behavior of these IF polymers when subjected to small and large deformations. A deformation is considered to be large when the viscoelastic moduli of the polymer become a function of the deformation amplitude, which defines the onset of the nonlinear regime (34). We first measured the elastic modulus of IF polymers after three cycles of oscillatory shear deformation as the ratio of the maximum measured stress to the maximum imposed strain (see “Experimental Procedures”). As expected, the wild-type keratin, mutant keratin, and vimentin IF polymers all display elastic moduli that are independent of the strain amplitude for small deformations (Fig. 3). All three polymers seemingly soften past a yield strain $\gamma$ of about 10% and display liquid-like properties ($G' < G''$) at $\gamma > 200\%$ (Fig. 3). This yield strain is similar to that obtained for F-actin suspensions under comparable conditions (41). Again, the nearly identical behavior of the three types of samples suggest that wide variations in the length of individual filaments does not significantly influence the behav-

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Fig. 3. Strain dependence of the elastic (filled symbols) and loss moduli (open symbols) of 1 mg/ml suspensions of K5-K14 (circles), K5-K14PN150 (triangles), and vimentin (squares) filaments.

The currently accepted models stipulate that the plateau modulus of an entangled solution of a flexible polymer (such as IFs) varies with concentration as $G \sim C^{2.25}$ (44), whereas the plateau modulus of semiflexible and rigid polymers varies with concentration as $G \sim C^{1.4}$ (45) and $G \sim C^1$ (44). In these models steric interactions, i.e. topological constraints due to entanglements between individual polymers, are the only mechanisms by which the relaxation of polymers in solution is impeded; all other possible nonsteric interactions are ignored.

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The strain for eight cycles of shear. Stress and strain are zero at time 0. Polymeric strain hardens, resisting further deformation (Fig. 4). During the first cycle of shear, the stress initially increases linearly with the imposed strain, i.e., the behavior of the gel is linear. However, the stress soon increases faster than the imposed strain, i.e., the keratin polymer strain hardens, resisting further deformation (Fig. 4). The short K5-K14 filament suspensions behave in a manner similarly to that of wild-type K5-K14 in this assay (Fig. 5), implying that at relatively high polymer concentration (1 mg/ml) this phenomenon does not depend upon filament length. On the other hand, the vimentin IF suspension shows a quick drop of its elastic modulus (41, 42).

In experiments involving large oscillatory deformations (Fig. 3), we noted that the wild-type keratin IF polymer consistently recovers its original storage modulus $G'$ and phase shift $\delta$ within 1 min after cessation of the shear strain (data not shown). Depending on the parameters of shear strain, the observed values for $G'$ after recovery were sometimes greater than the original (preshock) values. To investigate this phenomenon further, we conducted experiments in which a short-lived, steady deformation is imposed successively more than 20 times, each time for 5 s at a rate of 0.05 s$^{-1}$ (each equivalent to a cumulative strain of 25%). The time-dependent stress was monitored after each steady shear deformation (Fig. 5). We invariably found that as soon as the deformation is stopped, the K5-K14 IF polymer recovers its initial stiffness and often overshoots it. The elastic modulus typically increases by more than one order of magnitude after ~10 consecutive deformations and saturates thereafter (Fig. 5). The new plateau modulus attained after each deformation is stable, because there is no evidence for relaxation for as long as 20–30 min after cessation of the shear strain (data not shown). A build-up in stiffness is seen even when the K5-K14 suspension is subjected to larger deformations known to induce yielding (e.g., 0.3 s$^{-1}$ for 5 s), although we find that shear displacement and shear rate can influence the parameters of polymer recovery (data not shown).

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![Fig. 4. Time-resolved stress and strains in keratin filament suspensions. A, stress, $\sigma(t)$, induced in a 1 mg/ml suspension of K5-K14 filaments by an oscillating shear strain, $\gamma(t)$, of amplitude 100% and frequency 0.1 rad/s. B, time-dependent stress versus time-dependent strain for eight cycles of shear. Stress and strain are zero at time 0 (*)]. The arrows indicate the direction of shear.
and especially keratin IFs is in stark contrast to that of un-cross-linked F-actin, which takes >2–4 h to recover after a yield-inducing strain and which does not show an enhanced stiffness. 3 Although made remarkable by its uniqueness among biological polymers not subjected to external influences such as cross-linkers, this property of keratin IF suspensions is qualitatively similar to that previously described for associating polymers (43).3

**DISCUSSION**

We showed here that suspensions of pure epidermal keratin IFs and vimentin IFs behave like visco-elastic solids. When subjected to small deformations in the linear regime, keratin and vimentin IFs form a relatively stiff gel compared with other fibrous cytoskeletal biopolymers (e.g., F-actin and microtubules), as revealed by an exceptionally low phase shift between the input strain and the output stress. This behavior requires a significant degree of polymerization but is only partially dependent upon the length of individual polymers (see below). With increasing deformation the keratin and vimentin IF polymers show transient strain hardening but yield relatively rapidly. Electron microscopy studies in progress suggest that yielding is associated with the alignment of individual filaments along the axis of the imposed strain and is not due to filament breakage (data not shown). Furthermore, we find that keratin and vimentin IF polymer suspensions recover very rapidly upon cessation of a yield-inducing deformation and that, remarkably, the elastic modulus of the polymer can be built up to a significant extent depending on shear parameters and shear history. Collectively these data indicate that keratin and vimentin IFs display mechanical properties that are unique among the major known cytoskeletal polymers.

Provided that a simple set of criteria pertaining to the balance between polymer dimensions and polymer concentration are met, the visco-elastic modulus of a fibrous polymer is a composite of two types of energies, entropy and enthalpy (44). The entropic component is provided mainly in the form of steric effects between entangled polymers. Numerous studies have established that the viscoelastic properties of suspensions of F-actin and microtubules can be nearly entirely accounted for by steric interactions between entangled polymers (45, 47). In contrast, the three IF polymers tested here display the typical signature of networks whose properties cannot be entirely accounted for by steric interactions. In strong support of this interpretation are the larger-than-expected, frequency-independent modules of dilute, as well as overlapping solutions of keratin filaments (see below), and the observed relaxation time, which is much larger than that predicted for a dilute or overlapping suspension of rigid rod molecules such as that formed by K5-K14 at a concentration (1 mg/ml) protein concentration with those measured for K5-K14 at 0.05 mg/ml. Under such conditions we find that the elastic modulus of a wild-type keratin IF filament (typically 30–40 dynes/cm²) is ~10-fold larger than that of a gel made up of shorter K5-K14 IF filaments. Still, the length of individual polymers does not have as dramatic an impact in a keratin gel as it does for suspensions of F-actin (50). At another level, our data show that a significant degree of polymerization is required for IFs to display the properties of a visco-elastic solid material. This suggests that the nonsteric interactions taking place between individual polymers are stereospecific.

The findings and conclusions reported here are significantly yet incompletely related to those of three previous studies that made use of similar rheological instrumentation. Jamney and colleagues found that vimentin IFs (38) and neurofilaments (39) behave as strong viscoelastic gels whose properties are a weak function of polymer concentration and input strain frequency and that display strain hardening. These authors also found that neurofilaments are better able to resist deformation than vimentin IFs, a property that they ascribe to the formation of cross-bridges between individual neurofilaments that

3 D. Wirtz, unpublished data.

4 Our calculations assume that each K5-K14 filament, whose mass is ~6–8 nm wide and ~70–80 nm long by electron microscopy, are comprised of two overlapping sets of tetramer subunits (between 48 and 64 monomers total). The precise number of molecules depends on an accurate determination of the mass of individual K5-K14 filament, which is lacking at present.

5 This actually represents an underestimate, because G'(ω) → G', only when \( \omega >> (6 \tau_s)^{-1} \approx 550 \text{ rad s}^{-1} \), where \( \tau_s \) is the relaxation time of a K5-K14 filament (44).
involve the nonhelical tail domains of their heavy and medium protein subunits (39). In a separate study, Rogers et al. (40) confirmed that vimentin IFs behave like a relatively stiff viscoelastic solid and determined that the tail domain of vimentin significantly modulates the behavior of the polymer when subjected to large deformations. At odds with our conclusions, however, Janney et al. (50, 51) concluded that the behavior of vimentin IFs can be entirely accounted for by entropy when invoking polymer entanglement effects. Additional studies will be required to determine the source of these differences in interpretation. Our studies significantly extend these previous ones, in that we showed that: (a) suspension of keratin and vimentin IFs yield relatively rapidly but recover virtually instantaneously, in that we show that: (b) depending on the shear parameters, the elastic modulus of the keratin network (but not vimentin) can be built up to significantly larger values; and (c) nonsteric interactions are likely to contribute significantly to the visco-elastic moduli of IF suspensions in vitro.

Further studies are required to explain the elastic solid-like properties of a dilute suspension of short keratin IF polymers and to define the nature of the nonsteric interactions that contribute to the visco-elastic moduli of IF suspensions. Importantly, all the calculations and interpretations presented here are valid under the assumption that individual IF polymers are homogeneous in size (monodisperse) and in distribution when in solution. This notion needs to be tested experimentally, and we note that standard electron microscopy is clearly insufficient for that purpose (Fig. 1). Two major types of mechanisms, long range and short range, can be invoked to explain the gel properties of dilute suspensions of short keratin IF polymers. If these short polymers can interact with one another, then the formation of large domains or clusters could underlie the observed elastic properties because of the formation of percolated structures in which the large domains are interconnected in solution. This mechanism involves short range effects and implies that the suspensions of keratin IF polymers are homogeneous. If, on the other hand, IF polymers are homogeneous in their spatial distribution, then long range interactions (e.g. electrostatic effects) could account for the observed elasticity. We have not formally tested whether a dilute suspension of vimentin IFs, a polymer whose assembly in vitro is (at least partly) promoted by the addition of salt, displays the properties of a solid-like material, so that we cannot draw from our current data to speculate on the role of electrostatic interactions. Relatively simple assays can be applied to assess the distribution of individual molecules in an IF polymer suspension, and together with protein domain mapping experiments this should provide insights into the nature of the nonsteric contribution to the properties of IF gels.

A primary function of keratin and other types of cytoplasmic IFs in vivo is to endow cells with the structural resilience they need to withstand physiological doses of mechanical stress (see the Introduction). Accordingly IF networks are particularly well developed in tissues and cell types subjected to substantial mechanical forces, such as muscle and surface epithelia (20). Even though IFs are inherently mechanically resilient, it is doubtful that the values of elastic modulus obtained for suspensions of keratin or vimentin IFs can account for the ability of relevant cell types to cope with incident frictional forces. In that regard the lessons learned from the actin field are very useful. Whereas suspensions of pure F-actin display a weak viscoelastic modulus (e.g. \(G' = 10 \text{ dynes/cm}^2\); phase shift \(\delta \approx 25^\circ\); Ref. 41), addition of filament cross-linking proteins to F-actin invariably causes a dramatic increase in gel stiffness, up to values that are characteristic of those prevailing in the cell cortex in vivo (\(G' \approx 1000 \text{ dynes/cm}^2\); phase shift \(\delta \approx 10–15^\circ\); Refs. 46 and 52). By analogy, one may need to invoke accessory proteins to endow IF networks with a mechanical potential that is up to the functional challenges being faced in vivo. Cytoplasmic IFs are known to be firmly anchored at the plasma membrane (often at adhesion complexes) as well as near the surface of the nucleus (20, 21). In the cases of desmosome-mediated cell-cell adhesion and hemidesmosome-mediated cell-matrix adhesion in epithelial cells, some of the molecular effectors of this anchorage have been identified (20, 53–55). In addition, there are cytoplasmic proteins, such as plectin and bulbous perimembranous antigens isoforms, that have the ability to cross-link individual IFs to one another or to either F-actin or microtubules (20, 54, 55). It appears likely, therefore, that the organization of IFs into a pan-cytoplasmic network with a specific pattern of anchorage at multiple points is a key feature of their structural function in vivo.

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