Cross-Linking Molecules Modify Composite Actin Networks Independently

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While cells make use of many actin binding proteins (ABPs) simultaneously to tailor the mechanical properties of the cytoskeleton, the detailed interplay of different ABPs is not understood. By a combination of macrorheological measurements and confocal microscopy, we show that the ABPs fascin and filamin modify the structural and viscoelastic properties of composite *in vitro* actin networks independently. The outnumbering ABP dictates the local network structure and therefore also dominates the macromechanical network response.

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Cell shape, mechanics, and motility are determined by cross-linked and bundled actin networks. As in living cells many different actin binding proteins (ABPs) are present simultaneously, it is necessary to study their mechanical function in well-defined *in vitro* systems where the type and concentration of the ABP can be controlled [1]. While considerable progress has been made in understanding cytoskeletal networks that are cross-linked or bundled by individual ABPs such as fascin [2], filamin [3], or α-actinin [4], the interplay of different ABPs needs still to be addressed [5]. While at saturating concentrations a competitive behavior can be expected, it is *a priori* unclear how multiple ABPs interact in a concentration regime where sufficient binding sites on the actin filaments are present for each ABP offered. Matters are complicated by the fact that biochemical affinities of most cross-linkers are comparable in magnitude. At the same time, it has been shown that already the pure geometry of cross-linkers significantly influences the structure and with that the mechanical response of such networks [6]. For short cross-linkers such as fascin, a bundling transition has been observed—where above a critical concentration networks are composed of straight cross-linked bundles [2]. In contrast, filamin creates networks whose constituents are mostly curved and branched bundles. The observed structural differences manifest themselves in distinct mechanical signatures—not only in the concentration dependence of the static plateau modulus but also in the viscoelastic frequency spectrum and the nonlinear response.

Here we show that fascin and filamin modify the viscoelastic properties of composite actin networks independently: By confocal microscopy and macrorheology, we demonstrate that composite actin-fascin-filamin networks combine the typical structural and mechanical properties that are observed for each pure cross-linked system, i.e., actin-fascin and actin-filamin networks, respectively. This can be rationalized by considering the modification of the microscopic network properties by the respective cross-linking molecules—both cross-linking molecules contribute to the bundle structure as well as to the interconnectivity of the bundles.

G-actin is obtained from rabbit skeletal muscle and prepared as described before [7]. The average length of the actin filaments is controlled to 21 μm using gelsolin obtained from bovine plasma serum following Ref. [8]. Muscle filamin was isolated from chicken gizzard and further purified as reported in Ref. [9]. Recombinant human fascin was prepared by a modification of the method of Ref. [10] as described by Ref. [11]. Actin networks with distinct amounts of the ABPs fascin and filamin are investigated, tuning the molar ratio R of the respective ABP to actin $R = c_{\mathrm{APB}}/c_a$ over more than three decades up to $R = 0.2$. Polymerization is initiated by adding a $10x$ F-buffer as described in Ref. [7]. The viscoelastic response of actin-ABP networks is determined by measuring the frequency-dependent viscoelastic moduli $G'(f)$ and $G''(f)$ with a stress-controlled rheometer (Physica MCR 301, Anton Paar, Graz, Austria) over a frequency range of three decades following Ref. [7]. To observe the network structures, actin was labeled with phalloidin-TRITC (Sigma). Confocal images were taken with a confocal microscope (TCS SP5, Leica, Wetzlar, Germany).

Confocal images reveal that actin bundles formed by fascin are highly straight in shape [Fig. 1(a)]. In contrast, actin bundles formed by filamin are strongly curved and exhibit branches rather than simple cross-links as observed for fascin [Fig. 1(b)]. Composite actin-fascin-filamin (a-f-f) networks show a mixed morphology with straight and curved bundles appearing simultaneously. Branching points and putative cross-links coexist [Fig. 1(c)]. Even individual composite bundles combine characteristics of both fascin and filamin bundles: Straight bundles branching out [arrow in Fig. 1(c)] are observed as well as curved bundles showing kinks. The latter are due to the enforced hexatic order of actin filaments induced by fascin [12]. Evidently, a-f-f mixtures form homogeneous composite networks without any observable phase separation.

Actin networks formed by either fascin or filamin alone not only exhibit obviously unlike network structures but also show distinct frequency dependencies in their viscoelastic properties. The storage modulus $G'(f)$ of a pure fascin bundle network exhibits a plateaulike region at

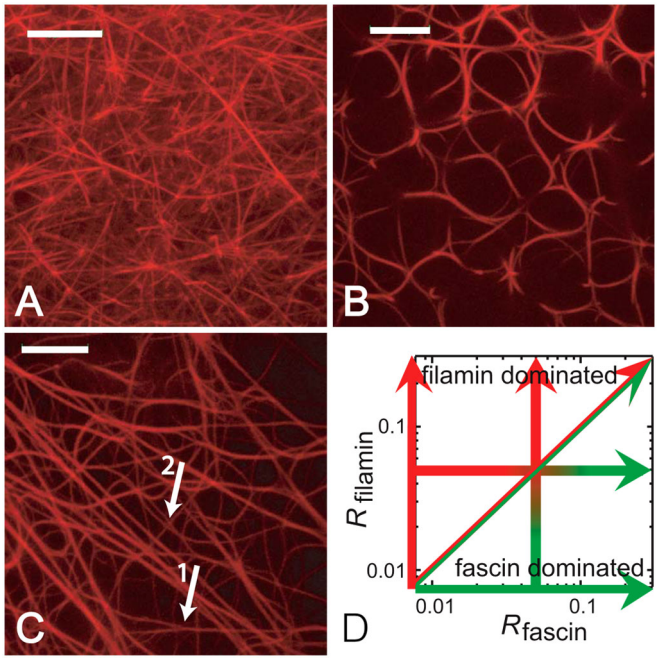

FIG. 1 (color online). Confocal images (projections of ≈ 5 μm height) of a pure actin-fascin network ($R_{fas} = 0.05$) (A), a pure actin-filamin network ($R_{fil} = 0.1$) (B), and a composite network ($R_{fas} = 0.01$, $R_{fil} = 0.1$) (C). Arrow 1 indicates a characteristic branching point of a straight bundle, which is not observable in pure fascin networks. Arrow 2 indicates a putative cross-link point which is extremely rare in pure filamin networks. $c_a = 9.5$ μM for all networks shown; scale bars denote 10 μm. A qualitative phase diagram of composite a-f-f networks is depicted in (D). The arrows represent the series of experiments conducted in this study.

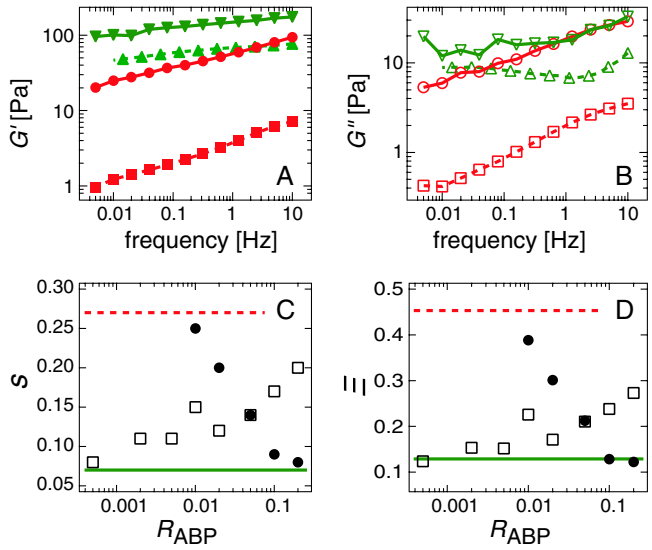

FIG. 2 (color online). The viscoelastic frequency response of actin-ABP networks ($c_a = 9.5$ μM) is dominated by the abundant ABP. The shape of $G'(f)$ (A) and $G''(f)$ (B) is similar for pure networks (one ABP, dashed lines) and composite networks (two ABPs, solid lines) where the respective ABP is present at a dominating concentration. Red denotes networks dominated by filamin: $R_{fil} = 0.2$ (squares), $R_{fil} = 0.2$ and $R_{fas} = 0.05$ (circles); green denotes mechanical dominance of fascin: $R_{fas} = 0.2$ (upright triangles), $R_{fil} = 0.05$ and $R_{fas} = 0.2$ (upside-down triangles). (C),(D) Steepness s and loss factor Ξ as a function of the cross-linker concentration. Open squares denote increasing R_{fil} in the presence of $R_{fas} = 0.05$; closed circles denote increasing R_{fas} in the presence of $R_{fil} = 0.05$. The lines represent the values of pure actin-fascin (solid) and actin-filamin (dashed) networks with the respective molar ratio $R_{ABP} = 0.2$.

intermediate frequencies: The modulus changes only about a factor of 2 within the measured frequency range (0.01–10 Hz). In contrast, the storage modulus of actin-filamin networks varies within the same frequency range up to about one decade [Fig. 2(a)]. Fitting the storage modulus with a power law $G'(f) \sim f^s$ over the whole frequency range measured here gives s as a quantity for the steepness of $G'(f)$. Moreover, the loss moduli of the pure systems significantly differ: Because of unbinding events occurring between distinct bundles, the loss modulus $G''(f)$ of actin-fascin networks shows a well-defined minimum [13]. However, such a minimum in the viscous dissipation is not observable for actin-filamin networks, which can be attributed to the fact that in these networks the bundles are branched rather than cross-linked. Thus, cross-linker unbinding might become mechanically relevant only at much longer time scales or high external forces as many unbinding events have to occur simultaneously in one bundle to rupture branches—resulting in a plastic deformation of the network. Consistently, very large nonlinear moduli can be reached by filamin networks even though the linear moduli are quite small [Fig. 4(b)]. A characteristic quantity describing the relative magnitude of dissipated energy to stored elastic energy at intermediate frequencies is given by the loss factor $\Xi = \frac{G''}{G'}$(0.16 Hz).

In this study, the concentration of one or both ABPs is varied up to $R = 0.2$ [Fig. 1(d)]. In this way, significant regions in the phase diagram of a-f-f networks can be explored. In order to avoid saturating concentrations where competitive effects might come into play, it has to be assured that the actin network provides enough binding sites for all ABPs offered. Therefore, the total number of *bound* ABPs per actin monomer R^*_{tot} is a crucial parameter to be controlled. $R^*_{tot} = R^*_{fas} + R^*_{fil} < 0.25$ for all networks observed here [14]—except for $R_{fas} = R_{fil} = 0.143$ [Fig. 3(c)]. As in pure actin-fascin bundle networks no saturation effects are observable below $R^*_{fas} = 0.25$ [12], competitive binding effects between fascin and filamin can be neglected in the concentration regime investigated here.

In order to investigate the viscoelastic properties of composite a-f-f networks, two series of experiments are conducted where the concentration of one ABP is kept constant at $R = 0.05$ while the concentration of the other ABP is increased from $R = 0$ to $R = 0.2$ (complete data shown in the supplementary material [14]). Raising R_{fas} in the presence of filamin results in a continuous flattening of the storage modulus $G'(f)$ and therefore a decrease of the storage modulus steepness s [Fig. 2(c)]. At the highest

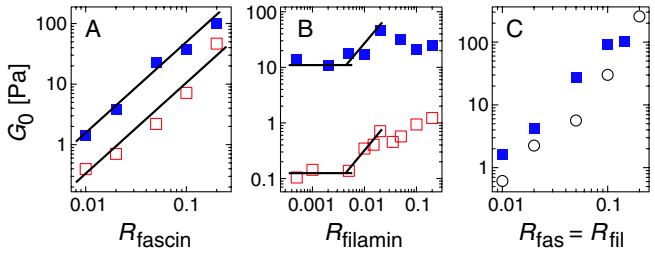

FIG. 3 (color online). G_0 as a function of c_a for pure (open squares) and composite (solid squares) actin networks. (A), (B) The concentration of one ABP is kept constant at $R = 0.05$ while the other one is increased. The lines represent scaling laws $G_0 \sim R_{fas}^{1.5}$ (A) as well as guides to the eye (B). (C) Both ABP concentrations are increased simultaneously ($R_{fil} = R_{fas}$). The increase of G_0 is predictable based on the data of the pure systems shown in A and B (open circles).

fascin concentration investigated ($R_{fas} = 0.2$), the shape of $G'(f)$ and therefore s is nearly identical to what is expected for a pure actin-fascin network with $R_{fas} = 0.2$ [Figs. 2(a) and 2(c)]. The loss modulus $G''(f)$ is also flattening with increasing R_{fas} until at $R_{fas} = 0.1$ a distinct minimum appears. With increasing R_{fas}, the loss factor decreases to a value resembling that of a pure fascin network [Fig. 2(d)]. These findings are consistent with results obtained for composite actin-fascin-α-actinin networks [5], where s as well as Ξ of the composite networks were found to have intermediate values compared to those of pure networks. Analogous experiments with a constant $R_{fas} = 0.05$ and increasing amounts of filamin ($R_{fil} = 0$ up to $R_{fil} = 0.2$) show complementary results: As R_{fil} is increased, the minimum in the loss modulus continuously disappears, Ξ increases [Fig. 2(d)], and the elastic modulus becomes gradually steeper [Fig. 2(c)]. At the largest filamin concentration investigated ($R_{fil} = 0.2$), both viscoelastic moduli $G'(f)$ and $G''(f)$ qualitatively reproduce the frequency behavior of a corresponding pure actin-filamin network [Figs. 2(a) and 2(b)]—but at higher absolute values. In summary, one ABP which is present at a distinct higher concentration than the other ABP dominates the microstructure of the network and with that the shape of the viscoelastic frequency spectrum of a composite network [Fig. 1(d)]. Yet the absolute values of both moduli are shifted to higher values.

An apparent plateau modulus $G_0 = G'(10\ \text{mHz})$ is defined to quantify the contribution of the two ABPs fascin and filamin to the elasticity of the composite network. Increasing R_{fas} in the presence of $R_{fil} = 0.05$ results in an enhancement of the network elasticity $G_0 \sim R_{fas}^{1.5}$ [Fig. 3(a)], which is exactly the same scaling relation as obtained for pure actin-fascin networks [2] (see also [14]). Similarly, the dependence of G_0 on R_{fil} is akin for both pure actin-filamin and composite a-f-f networks [Fig. 3(b)]: The plateau modulus hardly increases at low filamin concentrations, whereas between $R_{fil} = 0.005$ up to $R_{fil} = 0.02$ it increases about a factor of 5. Above $R_{fil} = 0.02$, a decrease of the static network elasticity is observed, which is caused by heterogeneities in density observable above this concentration of filamin. Yet, as s and Ξ are behaving well in this regime, the shape of the frequency spectra $G'(f)$ and $G''(f)$ is hardly affected by these heterogeneities. These findings show that fascin and filamin modify the network elasticity of the composite network independently as the mechanical enhancement caused by one ABP is unaffected by the presence of the other one. The observed independent modification suggests that the plateau modulus factorizes to functions which each solely depend on the concentration of a single ABP: $G_0(R_{fas}, R_{fil}) = \frac{1}{G_{0a}} G_0^{fas}(R_{fas}) G_0^{fil}(R_{fil})$, where G_{0a} denotes the plateau modulus of a pure actin solution. To further test this hypothesis, the concentrations of both ABPs fascin and filamin are increased simultaneously ($R_{fil} = R_{fas}$). The resulting strong increase of the network elasticity closely matches what is expected concerning the suggested factorization in combination with the already measured dependencies $G_0^{fas}(R_{fas})$ and $G_0^{fil}(R_{fil})$ [Fig. 3(c)]. Surprisingly, this holds true even at very high R_{fil}—despite occurring heterogeneities.

Already the linear response regime provides evidence for the independent behavior of fascin and filamin. However, further confirmation might be given by analyzing the nonlinear properties of composite a-f-f networks. The inherently pronounced differences in the nonlinear response of either fascin or filamin networks are ideally suited to test the proposed independent modification of the mechanical properties of composite networks by one distinct ABP. The nonlinear behavior is studied best with a constant shear rate $\frac{d\gamma}{dt}$ to minimize creep artifacts during the measurement [15]. From the recorded stress-strain relation $\sigma(\gamma)$, the differential modulus $K(\gamma) = \frac{\partial \sigma}{\partial \gamma}$ is calculated [15]. In pure actin-fascin networks, a continuous decrease in the degree of strain hardening is observed as a function of increasing R_{fas} [Fig. 4(c)]. This is attributable to forced unbinding events occurring between distinct fascin bundles [13]. However, the branched bundle structures formed by filamin give rise to a qualitatively different nonlinear behavior as strain hardening can be observed over the whole concentration regime in pure actin-filamin networks. In detail, within this strain-hardening regime K follows a power law $K \sim \gamma^x$. At the protein concentrations investigated here, the nonlinear exponent $x \approx 4$—describing the degree of strain hardening—is independent of R_{fil} [Fig. 4(d)]. Interestingly, this influence of fascin and filamin on the nonlinear response is conferrable to composite networks: Adding fascin to actin-filamin networks lowers the degree of strain hardening [Fig. 4(c)]. In contrast, adding filamin to actin-fascin networks again results in a constant degree of hardening [Fig. 4(d)]. Because of the presence of fascin, the nonlinear exponent $x = 1.1$ is clearly lower than the corresponding value for pure actin-filamin networks. This independent tunability of the nonlinear response underlines the independent modification of

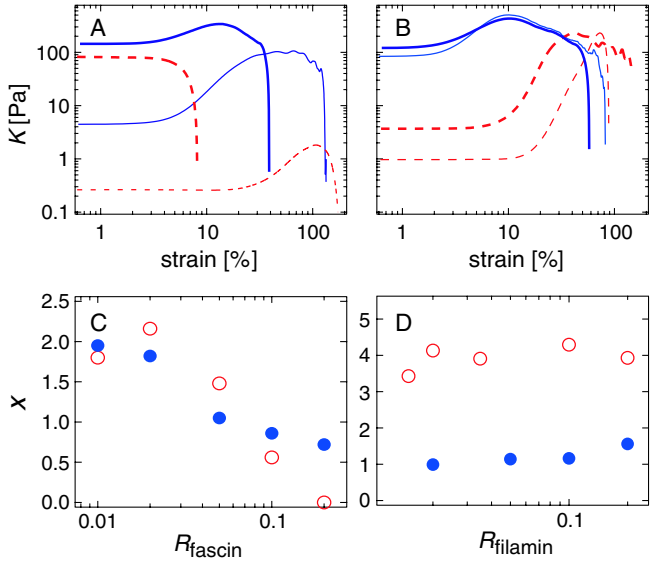

FIG. 4 (color online). (A) Increasing R_{fas} ($R_{fas} = 0.01$: thin lines; $R_{fas} = 0.2$: bold lines) decreases the degree of strain hardening in pure actin-fascin (red, dashed lines) as well as composite a-f-f networks ($R_{fil} = 0.05$: blue, solid lines). (B) The degree of strain hardening is unaffected by filamin ($R_{fil} = 0.02$: thin lines; $R_{fil} = 0.2$: bold lines) for pure actin-filamin (red, dashed lines) as well as composite a-f-f networks ($R_{fas} = 0.05$: blue, solid lines). (C),(D) x is depicted for increasing cross-linker concentrations for pure (open circles) and composite networks (solid circles). (C) x is shown as a function of R_{fas} for pure actin-fascin networks ($R_{fil} = 0$) and composite networks ($R_{fil} = 0.05$). (D) x is shown as a function of R_{fil} for pure actin-filamin networks ($R_{fas} = 0$) and composite networks ($R_{fas} = 0.05$). Strain rates of $\frac{d\gamma}{dt} = 6.25\%/\mathrm{s}$ are used for the pure fascin data shown in (A) and (C); all other data are acquired at $\frac{d\gamma}{dt} = 12.5\%/\mathrm{s}$.

the mechanical properties of composite networks by fascin and filamin as already observed in the linear regime.

In conclusion, composite a-f-f networks provide an enormous mechanical adaptability as tuning the concentrations of either ABP allows for adjusting a wide range of linear and nonlinear viscoelastic properties. A-f-f networks combine structural and mechanical properties of pure actin-fascin and pure actin-filamin networks. Both cross-linking molecules modify the linear and the nonlinear viscoelastic properties independently. The abundant cross-linker dominates the bundle configuration and the network microstructure—resulting in a macromechanical dominance in the composite network. The presence of another cross-linker which is distinctly less prominent in density only results in an offset in the viscoelastic properties. The mechanical and structural modification of the network by increasing amounts of a given cross-linker remains the same: The bundle microstructure as well as the interconnectivity of the bundles is modified in the composite a-f-f network exactly the same way as in pure actin-fascin networks and pure actin-filamin networks, respectively—depending on which ABP concentration is tuned. This demonstrates that different actin binding proteins can fulfill their mechanical function independently of each other without inducing any phase separation—not even on the local scale of single bundles. The described independent modification of the network elasticity is in marked contrast to a proposed synergistic enhancement of the static network elasticity by fascin and α-actinin [5]. However, in that study, the elastic modulus was found to be almost independent from the fascin concentration for $R_{fas} > 0.02$—in sharp contrast to our findings presented here and elsewhere [2]. The strikingly simple independent behavior confirms a reductionist's approach of studying *in vitro* networks that are cross-linked with only one single ABP: This method allows for the prediction of the dominating properties of more complex composite networks. In fact, a composite *in vitro* network with $R_{fil} = R_{fas} = 0.05$ reproduces the generic power law behavior [14] observed for living cells [16], which might simply be a result of structural heterogeneities and complicated cross-linker dynamics. However, the structural diversity of composite actin networks as well as the independent mechanical modification of the network by single ABPs need further theoretical attention. The observed mechanical redundancy and adaptability of composite actin networks are yet another possibility for cells to tailor their physical properties according to their needs.

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