Polymorphism of Cross-Linked Actin Networks in Giant Vesicles

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Actin networks cross-linked by natural linkers α-actinin and filamin are generated in giant vesicles by polymerization through ionophore-mediated influx of Mg^{2+}. α-actinin induces the formation of randomly linked networks at 25 °C which transform at <15 °C into spiderweblike gels or ringlike bundles depending on the vesicle size. Muscle filamin forms ringlike structures under all experimental conditions which can supercoil by subsequent Mg^{2+} addition. The polymorphism is rationalized in terms of recent models of bivalent ion coupled semiflexible polyelectrolytes and by considering the topology of the linkers.

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The actin-based cytoskeleton plays a central role for the regulation of a manifold of cellular processes: cell locomotion, phagocytosis, intracellular transport, and adhesion. For this purpose, the structural organization and dynamics of actin networks are manipulated by various actin binding proteins, such as filament severing proteins, monomer sequestering proteins, and cross-linkers. The cross-linkers are distinguished by two main features: the structure and the binding constant. It is often assumed that the former feature determines the organization of the actin gels which can form random networks with orthogonal orientation of two coupled filaments, ramified networks with branchlike knots, and arrangement of bundles [1]. Experimental evidence for this postulate are rare [2], and the rich polymorphism of actin gels could also be controlled by the association-dissociation equilibrium of the actin-cross-linker complex [3]. Up to the present most studies on actin gel formation were performed in bulk solutions [3], while under natural conditions (e.g., in cells) the dimension of the reaction space is of the order of the persistence length. This constraint could affect the structure of the network formed during polymerization [4], similar to the case of microtubules in vesicles [5].

To elucidate the effect of spatial constraints and the chemical and structural properties of the cross-linker on the structure of cross-linked actin gels, we studied the reconstitution of actin networks in giant vesicles of phospholipid-cholesterol mixtures. α-actinin and filamin were chosen as prototypes of weak cross-linkers exhibiting binding energies $\Delta H \sim k_B T$, thus forming dynamic networks. The structures were observed by confocal, epifluorescence, and phase-contrast microscopy. The spatial confinement allows one to visualize the structures more clearly than in the bulk solutions where fine structures are usually masked by the fluorescent background. Moreover, our system enables the perturbation of preformed structures by changing the Mg^{2+} content.

Actin was prepared from rabbit skeletal muscles with an additional gel filtration step in order to remove residual cross-linkers (see references in [6]). α-actinin was prepared from smooth muscle of turkey gizzard muscle [7]. Muscle filamin was isolated from chicken gizzard, and further purified [8]. All proteins were dialyzed against G*-buffer (2 mM Tris, 1 mM ATP, 0.05 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.005% NaN_3), a buffer adapted for G-actin and optimal vesicle electroswelling [6].

The preparation of actin containing vesicles has been described in detail previously [6]. In brief, we used dimyristoyl-phosphatidyl-choline lipids mixed with an $x = 0.17$ molar fraction cholesterol, doped with 2.5 mol % Dimyristoyl-phosphatidyl-ethanolamine-poly-ethyleneglycol 2000 (Avanti Polar Lipids, Birmingham, AL) and with 2.5 mol % ionophore A23187 (Sigma). With this membrane composition, nonspecific attraction between F-actin and the membrane is prevented [9]. Giant unilamellar vesicles (diameters from 5 to 30 μm) are formed by electroswelling. The intravesicular solution contained actin (from 2 to 10 μM), 110 mM sucrose, and either filamin (linker dimer-to-actin molar ratio $1{:}100 \leq r_{CA} \leq 1{:}30$) or α-actinin ($1{:}20 \leq r_{CA} \leq 1{:}10$) (total molarity 120 mM). The vesicles were sedimented in an Hepes buffer supplemented with glucose (total molarity 140 mM). Actin polymerization was initiated by transfer of Mg^{2+} ions into the vesicle through ionophores. The vesicles were observed at various temperatures, and for fluorescence, filamentous actin was labeled with rhodamine-phalloidin (Sigma) present in the initial solution at a ratio to actin monomers of 1:3.

The encapsulated actin/α-actinin networks (cross-linker to actin molar ratio $r_{CA} = 1{:}10$) undergo a transition from an isotropic network (with actin filaments preferentially oriented perpendicular to each other) to a bundled state if the polymerization is performed at high temperature (25 °C) and the vesicles are then cooled to 10 °C. At high temperature (25 °C) the network appears rather homogeneous on the resolution of the microscope [Fig. 1(a)]. This finding is consistent with viscoelastic studies of bulk actin/α-actinin networks at high temperature showing only a small increase of the viscoelastic impedance relative to entangled networks [3]. The network exhibits some heterogeneities if one is close to the

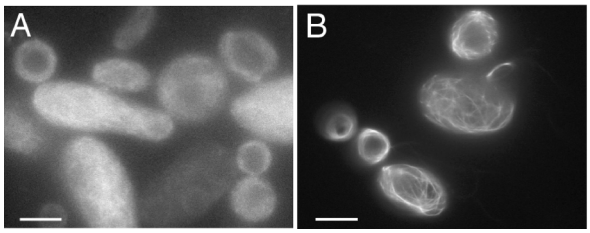

FIG. 1. Epifluorescence micrographs of structural change of actin/α-actinin networks at decreasing temperature (5 μM actin, α-actinin-to-actin ratio $r_{CA} = 1{:}10$). (a) At 25 °C, an isotropic network is formed with slight heterogeneities. (b) At 4 °C, the filaments have collapsed into bundles. (a) and (b) were taken at different areas on the same sample. Scale bar: 10 μm.

percolation transition [3] and because the filaments tend to accumulate close to the membrane of small vesicles to reduce their bending energy [9]. At decreasing the temperature below 15 °C, the network collapses by bundling of the actin [Fig. 1(b)]. Two types of structures appear. In vesicles of diameter smaller than 12 μm, rings are formed [Fig. 2(a)]. For vesicles of diameter larger than 12 μm, spiderweblike networks form, composed of thin bundles which cross or interconnect at starlike knots [Fig. 2(b)]. The fraction of vesicles containing bundled structures increased monotonically when the temperature decreased very slowly (1 °C per hour) from 15 to 5 °C. For smaller ratios $r_{CA} \leq 1{:}20$, bundles appear only after annealing for several days at 4 °C. At increasing the temperature again to 25 °C (with $r_{CA} = 1{:}10$), part of the bundles remain stable for a long time (> 24 h) but the fraction of vesicles containing bundles was found to decrease from 60% to 25% within 24 h, strongly suggesting that the bundling transition is reversible despite a strong hysteresis, due to a kinetic hindrance of the reverse transition.

In order to test the effect of filament length on bundling, the severing protein severin was added at a severin-to-actin ratio 1:100 (filament contour length of 0.3 μm [9]). At 4 °C, 80% of the vesicles do not contain large bundled structures. Some large vesicles (diameter > 15 μm) contain disorganized and mobile assemblies of short bundles, while some small vesicles (diameter <9 μm) contain again rings.

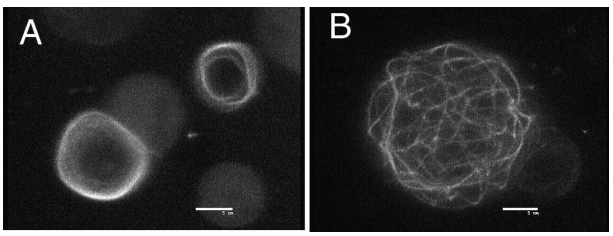

FIG. 2. Dependence of actin/α-actinin network structure on vesicle size. The 3D reconstructions of networks by confocal fluorescence microscopy ($r_{CA} = 1{:}10$, temperature 4 °C). (a) Examples of rings obtained in vesicles of diameter $d <$ 12 μm. (b) Spiderweblike networks formed in vesicles of diameter $d > 12$ μm [10]. Scale bar: 5 μm.

The muscle filamin induces the formation of bundles under most conditions, irrespective of the procedure of polymerization. Thus, at high actin concentrations ($c_A \sim$ 10 μM), spontaneous formation of actin bundles is observed even in the absence of Mg^{2+} for $r_{CA} \leq 1{:}100$ both in the bulk and within vesicles. At lower actin concentrations ($c_A \sim 3$ μM), polymerization is observed only after influx of Mg^{2+}. At low cross-linker density ($r_{CA} =$ 1:100), short slightly bent bundles are formed while the bulk of the actin is either entangled or weakly polymerized (not shown). The latter is suggested by the finding that the plateau modulus is only slightly increased [11]. At higher filamin contents ($r_{CA} = 1{:}30$), the bundles form closed circular rings as shown in Fig. 3(a). These rings are flat, as revealed by confocal microscopy (not shown). Phase-contrast images strongly suggest that in general the closed rings do not adhere to the membrane, if they exhibit a smaller radius than the vesicle [Fig. 3(b)]. The formation of the rings is not affected by the addition of severin at $r_{CA} = 1{:}100$. In contrast to muscle filamin, the filamin-analog of *Dictyostelia* cells (called 120 kDa gelation factor; see the review in [8]) forms isotropic cross-linked networks at $r_{CA} = 1{:}30$ and $c_A \sim 5$ μM actin.

A most interesting effect arises after ionophore-mediated influx of 2 mM Mg^{2+} into vesicles containing flat rings of F-actin linked by muscle filamin ($r_{CA} =$ 1:30) which were generated by polymerization under Mg^{2+} free conditions. As shown in Figs. 4(a)–4(c), some supercoiled rings are formed. The twisting angle is increased further by decreasing the temperature to 4 °C [4(b) and 4(c)]. If Mg^{2+} is added by successive addition of 0.5 mM (up to 2 mM), the flat rings buckle and form half-moon-like structures [Figs. 4(d)–4(f)]. As suggested by comparison of fluorescence and phase-contrast micrographs, the actin bundles adhere most likely to the membrane in this last case.

The flat rings often exhibit small bending fluctuations as shown in the example of Fig. 5, which indicates that the filaments are loosely coupled within the bundle. In these cases it is possible to estimate the bending stiffness B by Fourier analysis of the fluctuations $u(t)$ in terms of the cylindrical harmonics $u(t) = \Sigma u_m(t) e^{m\phi}$ where ϕ is the

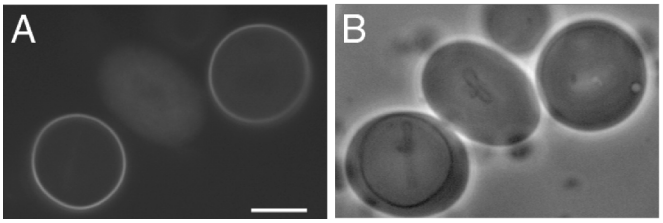

FIG. 3. Actin/filamin system in vesicles (10 μM actin) formed at $r_{CA} = 1{:}30$. (a) Fluorescence micrographs of ringlike structures. (b) Corresponding phase contrast images showing clearly that actin rings do not adhere to the vesicle membrane. Temperature 25 °C. Scale bar: 10 μm.

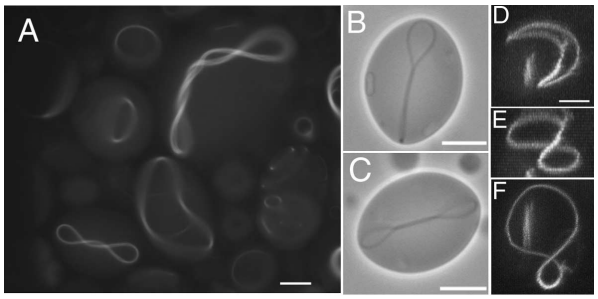

FIG. 4. Supercoiling and buckling of ringlike bundles in vesicles containing 10 μM actin and filamin at filamin-to-actin ratio of $r_{CA} = 1{:}30$, induced by the addition of 2 mM Mg^{2+}. (a) Fluorescence micrograph at 25 °C. (b),(c) Phase contrast micrograph of the same vesicle at 25 and 8 °C, respectively. Note that the vesicle is stretched since the length of the supercoiled ring is larger than the vesicle diameter [12]. Bar: 10 μm. (d)–(f) The 3D reconstruction of a buckled ring by confocal microscopy viewed under different angles. Bar: 5 μm.

azimut angle. Using equipartition theorem and [13] one finds

$$\frac{k_B T}{2} \sim \frac{B\pi}{R^3}(m^4 - 2m^2 + 1)\langle u_m u_m^* \rangle \qquad (1)$$

with R the radius of the ring. For the example of Fig. 5 the root mean square amplitudes $\langle u_m u_m^* \rangle$ of the second and third modes (24 ± 3 nm and 10 ± 2 nm, respectively) yield bending moduli of $B = 460\ \mu\text{m} \times k_B T$ and $B = 380\ \mu\text{m} \times k_B T$, respectively. The error of the amplitude measurement is ± 3 nm as estimated from the variance of the mode $m = 0$. By assuming that all actin is bundled by filamin, one can determine the total number of loops in the ring yielding $N = 65$ for the ring of Fig. 5 (10 μM actin, $R = 2.5\ \mu$m). The bending modulus of single actin filaments is $B_0 = 17\ \mu\text{m} \times k_B T$, and the above values of B thus would correspond to loop numbers $N = 27$ and 22, respectively, which are by a factor of 3 smaller than the above value. This discrepancy can be partially explained by assuming that only a fraction of the F-actin is bundled

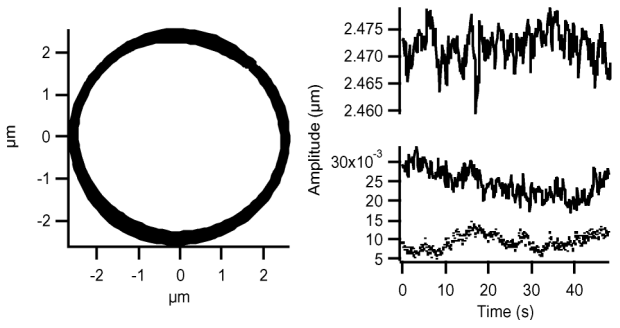

FIG. 5. Superposition of 400 traces of an actin/filamin ring taken every 117 ms. Time sequenced of amplitudes (in μm) of the cylindrical harmonics $m = 0$, $m = 2$, and $m = 3$ from top to bottom. The actin concentration was 10 μM.

by filamin. Indeed, by considering the residual fluorescence intensity in the center of the vesicles we estimate that only 50% of actin resides in the ring, thus reducing the number of loops to 30.

A most remarkable finding of our study of the formation of mesophases of actin gels by cross-linkers under biomimetic conditions is the striking difference between the forklike filamin and the rodlike α-actinin cross-linker or filamin of *Dictyostelium*, although all these linkers exhibit the same actin binding domains and similar binding constants [14]. Thus, α-actinin favors the formation of isotropic networks at low actin-to-cross-linker ratios r_{CA} and bundling at high r_{CA}, while muscle filamin induces bundling formation at all values of r_{CA} considered (similar to the behavior in bulk solution [11,15]). Our experiments suggest that ring formation by bundles is favored in confined spaces since in bulk solutions it is the exception rather than the rule at comparable linker or Mg^{2+} concentration [16]. In the case of α-actinin, the formation of closed rings in small vesicles upon cooling could be explained by the previous finding that the entangled network tends to form a fuzzy cortex [9]. In large vesicles, the initial F-actin distribution is homogeneous and thus leads to an homogeneous network of bundles. This is supported by the finding that the formation of large bundled structures is strongly reduced by shortening the filament with severin.

An intriguing finding is the supercoiling and buckling of the actin/filamin rings caused by Mg^{2+} influx into vesicles with preformed bundles or rings (cf. Fig. 4). According to studies of DNA, supercoiling occurs if the filaments exhibit a helical structure and if an additional nonequilibrium twist is imposed [12,17]. This provides some evidence that the actin/filamin bundles are twisted and that the intrinsic twist is changed by adsorption of Mg^{2+}. Interestingly, supercoiling of single actin filaments is also induced if one strand of F-actin slides over a second one by the action of myosin II [18].

The results can be understood in terms of recent statistical mechanical models of liquid crystal formation of polyelectrolyte rigid rods cross-linked by bivalent ions [19] which were developed to explain the formation of raftlike assemblies of Ca^{2+}-linked filaments by x-ray diffraction studies [20]. In the absence of linkers (entangled networks) the rods tend to orient in an orthogonal fashion (thus favoring isotropic network formation) due to the electrostatic interfilament repulsion energy. This effect could be small for natural cross-linkers which enable interfilament distances larger than the Debye screening length. Similarly, the translational entropy of the linkers is expected to favor random network formation since it is maximal if they interconnect the naturally occurring crossings of the filaments. For semiflexible filaments, the bundle formation by linkers is determined by the interplay of electrostatic repulsion between the rods and their bending elasticity [19]. The possibility of local filament bulging between two adjacent linkers

reduces the electrostatic energy at short distances while an effective repulsion arises at large separations. At medium linker density, the model predicts the lateral aggregation of the linkers into clusters separated by regions of decoupled filaments. This crossover regime could correspond to our frequent finding that the rings formed with α-actinin exhibit tightly packed and fuzzy regions [cf. Fig. 1(b)].

α-actinin is a rodlike antiparallel homodimer with actin binding sites at the opposite ends, while muscle filamin is a forklike homodimer with the two binding sites at one end which defines a symmetry plane. It is thus conceivable that the constraint concerning the orientation of two filaments to achieve optimal fitting is less stringent for α-actinin than for filamin. Because of the bifurcated structure of filamin, one arm would have to be rotated by 90° to allow optimal fitting of the binding site at orthogonal filament orientations. The torsional energy cost associated with this torsion is expected to favor bundling. Conversely, if the two binding sites bind to the same filament, the induced torque by the torsion of the filamin could favor twisting of single filaments and thus supercoiling of the bundles. Evidence for this model is provided first by the finding that other cross-linkers exhibiting forklike structures such as cortexilin from *Dictyostelia* cells [21] also favor bundling while the short filamin of *Dictyostelia* yields isotropic gels. Further evidence for the control of the structure of the actin gels by the topology of the linker is provided by the following observation: Streptavidin induces the formation of random networks of biotin labeled actin and does not form rings or large bundles (using 5 μM actin, 20% biotinylated actin, equimolar streptavidin; data not shown). It is, indeed, expected that the topology of the tetravalent linker streptavidin favor orthogonal filament orientation since two pairs of binding sites are oriented perpendicularly. Closed rings have, however, been reported in [16] at much lower concentrations of actin, which is expected to favor self-reaction of the filaments.

In cells actin-myosin rings play a central role for the pinching off of two daughter cells during cell division. Both filamin and alpha-actinin have been identified to be associated with ringlike bundles in some cells [22]. To our knowledge, such structures are observed for the first time *in vitro,* in the presence of natural cross-linkers.

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