

Actin-binding Protein Promotes the Bipolar and Perpendicular Branching of Actin Filaments

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ABSTRACT Branching filaments with striking perpendicularity form when actin polymerizes in the presence of macrophage actin-binding protein. Actin-binding protein molecules are visible at the branch points. Compared with actin polymerized in the absence of actin-binding proteins, not only do the filaments branch but the average length of the actin filaments decreases from 3.2 to 0.63 μm . Arrowhead complexes formed by addition of heavy meromyosin molecules to the branching actin filaments point toward the branch points. Actin-binding protein also accelerates the onset of actin polymerization. All of these findings show that actin filaments assemble from nucleating sites on actin-binding protein dimers. A branching polymerization of actin filaments from a preexisting lattice of actin filaments joined by actin-binding protein molecules could generate expansion of cortical cytoplasm in amoeboid cells.

In 1939, Lewis (16) speculated, on the basis of observations with the light microscope, that transformations between gel and sol states in the peripheral cytoplasm of macrophages direct the movements of this cell. Recent studies with cytoplasmic proteins isolated from macrophages and other cells have provided evidence for such transformations and some explanations for this phenomenon at the molecular level. Actin filaments are abundant in the peripheral cytoplasm of macrophages (1, 23), and molecules of actin-binding protein, which cross-link actin filaments into an isotropic gel (4, 7, 27, 28), are concentrated in the macrophage periphery (25). Therefore, cross-linking of actin filaments by actin-binding protein could be responsible for a gel state of macrophage peripheral cytoplasm. Gelsolin, a calcium-binding protein, effectively divides actin filaments in a calcium-dependent manner. This protein, by reversibly severing actin filaments between actin-binding protein cross-links, regulates the consistency of an actin lattice (26, 33, 34, 35).

The morphology of gels created by the addition of actin-binding protein to actin filaments when examined with the electron microscope after negative staining is virtually indistinguishable from that of solutions containing only actin filaments, even when actin-binding protein concentrations are 10 times higher than those required for the initial gelation of actin. High concentrations of actin-binding protein (molar ratio of actin-binding protein to actin of 1:20) result in the formation of cross-linked actin filament bundles visible in electron micrographs. Although actin-binding protein molecules can be recognized, cross-linking actin filaments in these bundles formed with high concentrations of actin-binding protein (8, 28), a

general picture of the junctions where cross-linking of actin occurs in actin gels formed with much lower actin-binding protein concentrations is difficult to obtain (8, 28). This is not surprising, because these gels are composed of actin polymers overlapping at random, and only a small but critical number of the filament crossings need be joined by molecules of actin-binding protein to cause gelation (6).

Actin filaments and monomers coexist in solution, the equilibrium disposition of the two states being determined by the ambient conditions of solvent and temperature (22). Because actin polymerized in the presence of actin-binding protein also gels (28), we reasoned that it might be advantageous to visualize the junctions between polymers linked early in the process of copolymerization. In this paper, we document that actin assembled in the presence of actin-binding protein forms branched structures with actin-binding protein molecules at the branching sites, and we provide evidence indicating that nucleation of filament growth is part of the branching process. Furthermore, we have determined the polarity of the actin filaments at branch points, and the results suggest an additional mechanism for movement of the macrophage cortex.

MATERIALS AND METHODS

Proteins

Actin-binding protein was purified from rabbit lung macrophages as described previously (7). Actin was isolated from the leg and back muscles of rabbits by the procedure of Spudich and Watt (24), except that 0.8 M KCl was substituted for 0.6 M KCl during the first polymerization cycle. G-actin was passed through a sterile 0.45- μm Millipore filter (Millipore Corp., Bedford, Mass.) and stored at 4°C in 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , and 2 mM

Tris-HCl, pH 8.0. The stored actin was gel-filtered on a Sephadex G-150 column in the same buffer to remove endogenous nuclei before measuring the effect of actin-binding protein on actin polymerization by flow birefringence. Heavy meromyosin was prepared by trypsin digestion (17) of myosin purified from rabbit back muscles.

Analytical Procedures

The flow birefringence and extinction angle of actin solutions in the presence and absence of actin-binding protein were measured at room temperature in an Edsall-type apparatus (Rao Instrument Co., Brooklyn, N. Y.) as described by Maruyama (19). Protein concentration was assayed by the Folin technique (18), using bovine serum albumin as the standard. The morphology of actin and of the structures formed by mixing G-actin with actin-binding protein were studied in the electron microscope by negative staining and low-angle rotary shadowing of the proteins. (a) For negative staining, protein solutions were applied as single drops to Formvar- and carbon-coated copper grids. G-actin alone or G-actin and actin-binding protein in buffer A of Spudich and Watt (24) were placed in small test tubes. 3 M KCl was added to give a final concentration of 0.1 M, the tubes were swirled with a Vortex mixer (Scientific Products Co., Div. American Hospital Supply Corp., McGaw Park, Ill.), and a drop of the solutions was transferred immediately to the surface of a grid. The protein solutions were then incubated for 10 min at room temperature, after which the liquid was removed by touching filter paper to the edge of the grid. The grids were washed twice by rinsing with 0.1 M KCl, 10 mM imidazole-HCl, pH 7.0, and negatively stained with 1% uranyl acetate. The stereospecific binding of heavy meromyosin (10) was used to determine the polarity of actin filaments on negatively stained preparations. Mixtures of actin and actin-binding protein were applied to grids, incubated, and washed as described above. One drop of heavy meromyosin, 0.2 mg/ml in 0.1 M KCl, 10 mM imidazole-HCl, pH 7.5, was applied to the grid, and the grids were then rinsed once with 0.1 M KCl, 10 mM imidazole-HCl and negatively stained with 1% uranyl acetate. The grids were examined and photographed in a Philips 200 electron microscope at an accelerating voltage of 60 kV. (b) For low-angle rotary staining, the rotary shadowing technique of Tyler et al. (31) was used to study the interaction of actin-binding protein with actin. G-actin was polymerized in the presence of actin-binding protein as described above. The protein solution was then diluted with 9 vol of 48% glycerol. This solution was then sprayed onto mica; the mica was dried under vacuum and rotary shadowed with platinum/carbon at a 4.5° angle as described by Tyler et al. (31), giving a heavy metal deposit of 0.7–0.8 nm. The replicas were examined in a Philips 301 electron microscope at 80 kV.

500 angles of intersection of actin filaments at branch points (actin alone) were measured (values between 0° and 90°) in electron micrographs by means of a protractor. A large number of side-to-side filament interactions were observed on the grids containing actin alone. When the regions of filament apposition were ≥ 50 nm in length, they were counted as zero angle intersections irrespective of the angles at which the filaments approached before becoming parallel. The length of actin filaments formed in the absence and presence of actin-binding protein was measured as previously described (7).

F-actin solutions, 1 mg/ml, were fragmented by sonication in a Sonicator cell disruptor (Heat Systems-Ultrasonics, Plainview, N. Y.) at a 6.5 power setting for 40 s.

RESULTS

Morphology of Actin Copolymerized with Actin-binding Protein

Actin and actin-binding protein were briefly copolymerized in 0.1 M KCl solution on grids, negatively stained, and viewed in the electron microscope. Under these conditions, branching actin filaments were the dominant structures (Fig. 1). Compared with control actin, the branching angles of the filaments had a remarkable perpendicularity (cf. Figs. 1B and 2). Two-thirds of the actin filaments branched at angles near 90° (Fig. 2B). Because, as amplified below, only short actin filaments were formed when polymerization was carried out in the presence of actin-binding protein, the increase in the number of perpendicular branches relative to control cannot be explained by an increase number of uncross-linked filaments overlapping at right angles because of their origin at perpendicular branches. Actin polymerized in the absence of actin-binding protein on grids had some tendency to intersect close

to right angles but much less so than did the copolymerizing material (Figs. 1B and 2A). In general, side-to-side interactions of the actin filaments were found in these controls (Fig. 1B). The perpendicular branches assumed three principal configurations. Filament segments intersected as L-shaped (large arrowheads), T-shaped (small arrowheads) or X-shaped junctions (double small arrowheads) (Fig. 1A). No preference for the direction of branching from either side of long filaments was apparent, nor was there any evidence of a regular spacing between branches.

The actin filaments formed in the presence of actin-binding protein were shorter in the electron microscope than the actin controls. Flow birefringence measurements showed that actin polymerized in this manner was completely polymerized within 5 min. The number average, $(L)n$, and the weight average lengths, $(L)w$, were $2.1 \pm 1.6 \mu\text{m}$ and $3.23 \mu\text{m}$ for the control actin, and $0.48 \pm 0.33 \mu\text{m}$ and $0.63 \mu\text{m}$ for actin polymerized in the presence of actin-binding protein, respectively, where $(L)n = (\sum n_i \ell_i) / (\sum n_i)$ and $(L)w = (\sum n_i \ell_i^2) / (n_i \ell_i)$, n being the number of particles and ℓ being the length of the particles. This finding suggested the possibility that perpendicular branching might occur more often when the filaments are short. To ascertain the effect of actin filament length on the morphology of interfilament junctions, actin filaments were fragmented by sonication, applied to grids, and viewed in the electron microscope. No difference in the morphology of actin filament interactions relative to control actin was found in the sonicated samples.

Particles with the structure of molecules of actin-binding protein (8, 31) were visible at actin filament branch points. Fig. 3 shows by rotary shadowing that branch points are held together by structures composed of long linear strands. The morphology of purified actin-binding protein dimers is shown in Fig. 3B. One such dimer cross-linking two actin filaments formed by the interaction of preformed actin filaments with actin-binding protein (Fig. 3C) is shown for purposes of comparison. As shown by comparison of strands found at branch points (Fig. 3A) with purified actin-binding protein, these strands have the same width as those of actin-binding protein molecules and appear to wrap around the actin filament. The resolution in negatively stained preparations is too low to obtain an adequate picture of these structures at branch points but inspection of the branch points in Fig. 1A shows densities at the junctions.

We determined the polarity of the branching filaments. Actin and actin-binding protein were copolymerized on a grid and then the filaments were decorated with heavy meromyosin, forming characteristic arrowhead structures on the actin filaments. Fig. 4 shows electron micrographs of short actin filaments branching from longer actin filaments after decoration with heavy meromyosin. The direction of filament growth from the T-shaped branch points was quantitated. 92% of the filaments were decorated such that the arrows pointed toward the branch points.

Actin-binding Protein Accelerates the Onset of Actin Polymerization

The effect of actin-binding protein on actin polymerization was studied by means of flow birefringence after addition of MgCl_2 to a final concentration of 1 mM, which causes polymerization at a relatively slow rate (22), and also after addition of KCl to a concentration of 0.1 M. G-actin remained

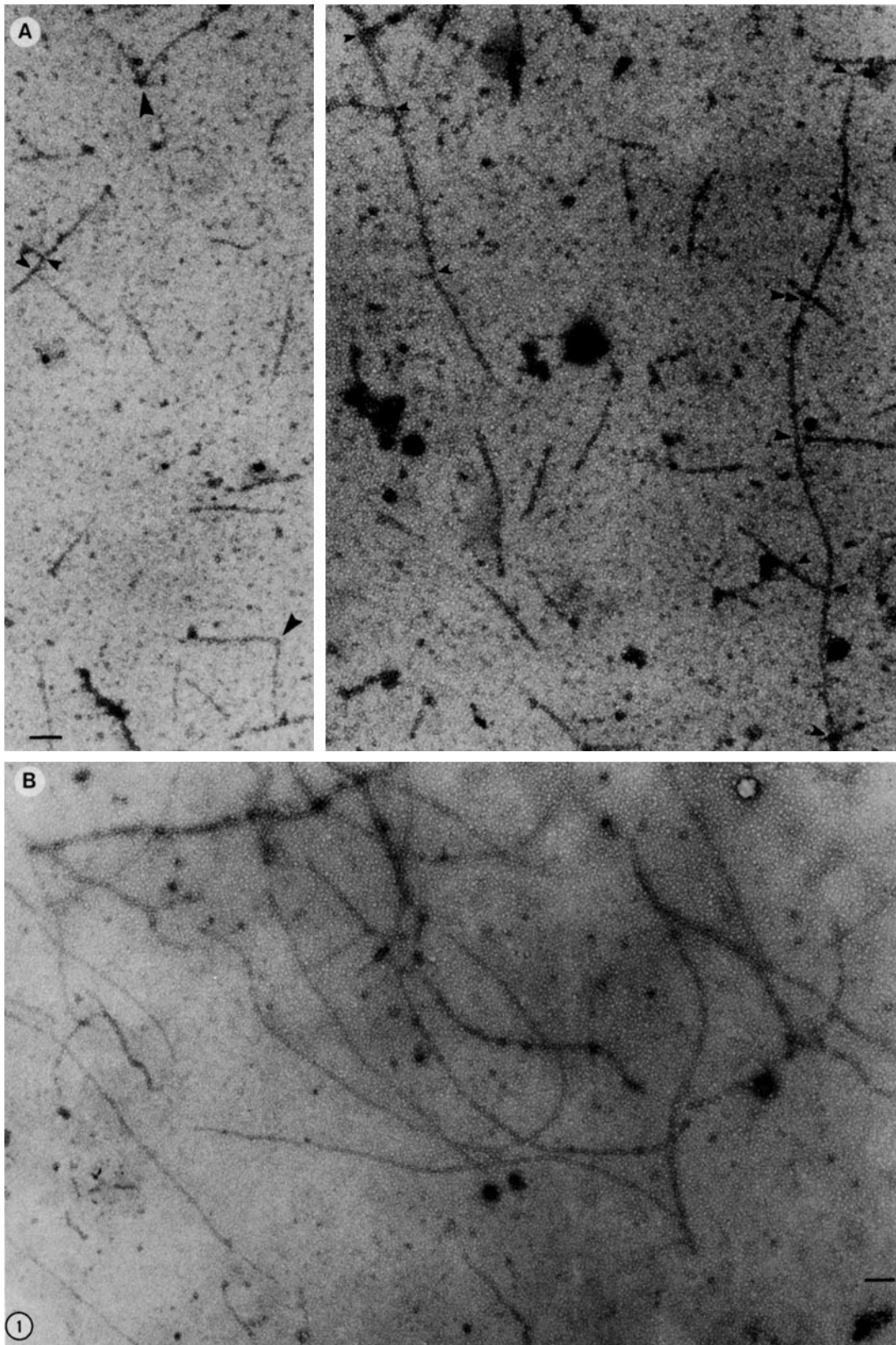


FIGURE 1 Representative electron micrographs of skeletal muscle actin, 75 $\mu\text{g}/\text{ml}$, polymerized in the absence (*B*) or presence (*A*) of 75 $\mu\text{g}/\text{ml}$ of actin-binding protein and negatively stained with uranyl acetate. Both proteins were placed on grids and polymerized as described in the text. (*A*) Low magnification showing the branching polymerization. The arrowheads designate actin polymer branching points. The smaller arrowheads indicate T- or X-shaped branches, as discussed in the text. The larger arrowheads show L-shaped branches. (*B*) Low magnification of 75 $\mu\text{g}/\text{ml}$ actin polymerized as in *A*, except in the absence of actin-binding protein. Bars, 0.2 μm .

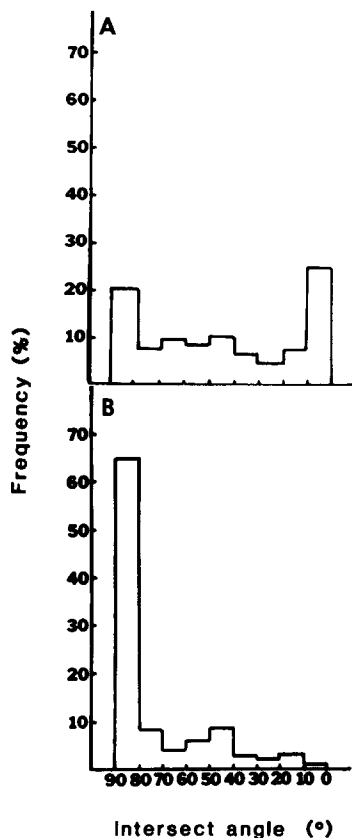


FIGURE 2 Histograms comparing (A) the angle of actin filament intersection for actin polymerized in the absence of actin-binding protein and (B) the angle of filament branching for actin polymerized in the presence of actin-binding protein. (A) Skeletal muscle actin, 75 $\mu\text{g}/\text{ml}$, was polymerized on a grid as described in the text. (B) Actin, 75 $\mu\text{g}/\text{ml}$, was polymerized in the presence of 75 $\mu\text{g}/\text{ml}$ of actin-binding protein on a grid. 500 filament intersections (A) or filament branches (B) were measured in each histogram.

monomeric for 60 min after the addition of MgCl_2 (Fig. 5). Polymerization of the actin under these experimental conditions began after 80 min and was completed within 180 min. As shown in Fig. 5, low concentrations of actin-binding protein (mole per mole of G-actin) accelerated the onset of actin polymerization. The final measured value for flow birefringence at 19 h for actin alone was 60° . The level of flow birefringence of actin solutions polymerized in the presence of actin-binding protein, 15 or 30 $\mu\text{g}/\text{ml}$, was decreased to 57° and 55° , respectively. Actin-binding protein, 30 $\mu\text{g}/\text{ml}$, also accelerated the onset of actin polymerization in 0.1 M KCl solution (Fig. 5B). The final value of flow birefringence was markedly reduced in the presence of actin-binding protein, and the extinction angle of actin was higher in the presence of actin-binding protein than in its absence, indicating shorter filaments in solutions containing actin-binding protein.

DISCUSSION

Actin filaments copolymerized with actin-binding protein form branching structures easily seen in the electron microscope. One of the most striking observations is the perpendicular branching of actin filaments in the presence of actin-binding protein. In the absence of actin-binding protein, side-by-side or perpendicular alignments of the actin filaments on grids are somewhat favored (Fig. 2A). The observation of actin filaments

joined in parallel is consistent with the previous documentation of birefringence (12), turbidity (20), and a rapidly sedimenting component (11) in actin filament solutions. The side-by-side associations may arise from the extreme length and helical structure of the actin filaments, the latter preventing the expression of electrostatic repulsions and favoring the juxtaposition of opposite charges in neighboring polymers (13). Filaments overlapping obliquely might tend to be drawn into parallel associations, whereas filaments intersecting at nearly perpendicular angles would tend not to be affected in this way, thereby accounting for the higher frequency of parallel rather than perpendicular interactions. The predominance of filaments branching perpendicularly in the presence of actin-binding protein suggests the presence of structural constraints at the branching points, most likely the molecules of actin-binding protein seen at these locations (Fig. 3). Electron micrographs of gels formed with filamentous actin and actin-binding protein also give the impression of perpendicular branching (8). However, the great length of actin filaments, the large number of filament crossover points in these gel networks, and the low concentrations of actin-binding protein used to gel actin solutions (molar ratio of 1 actin-binding protein to 1,000 actin monomers in filaments) makes the identification by negative staining of actin-binding protein molecules at filament interactions extremely difficult.

In principle, cross-linking of preexisting actin polymers can generate the same network structures as a branching condensation polymerization in which polymers grow by monomer addition at the cross-links (6). Hence, the branched networks of actin formed in the presence of actin-binding protein could result from the cross-linking of actin filaments by actin-binding protein and/or by a condensation polymerization in which the actin filaments assemble directly from each of the binding sites for actin filaments present on actin-binding protein molecules (8). Because each actin-binding protein molecule has two binding sites for actin filaments (8), growth of actin could proceed from either or both of these sites.

Previous work has shown that actin-binding protein cross-links actin polymers (4, 7, 8, 27, 28); the findings presented here indicate that a condensation polymerization can also take place. First, actin-binding protein accelerates the onset of actin polymerization in 1 mM MgCl_2 and 0.1 M KCl solutions, indicating that it acts as a nucleus for actin filament growth, a condition expected to increase the number of filaments and decrease filament length (14, 22). In agreement with this expectation, actin filaments assembled in the presence of actin-binding protein are markedly shorter than those polymerized in its absence. Second, the polarity of the actin filaments at branch points is consistent with filament growth originating at the branches and moving outward (9, 15, 32) in what has been called the "preferred" direction of growth. This kind of branching could proceed by several mechanisms. Filaments could grow in the "preferred" direction simultaneously from the opposite ends of the actin-binding protein subunits, leading to the L-shaped configurations seen in Fig. 1. If the branched actin filaments are not capped on the end by actin-binding protein molecules, some filament growth in the "unpreferred" direction would create T- and X-shapes. Filaments could also grow from one chain of actin-binding protein either before or after the other chain bound to a preformed filament, leading to the T- and X-shaped images.

By decreasing the average length of actin filaments, actin-binding protein causes the formation of a more highly branched

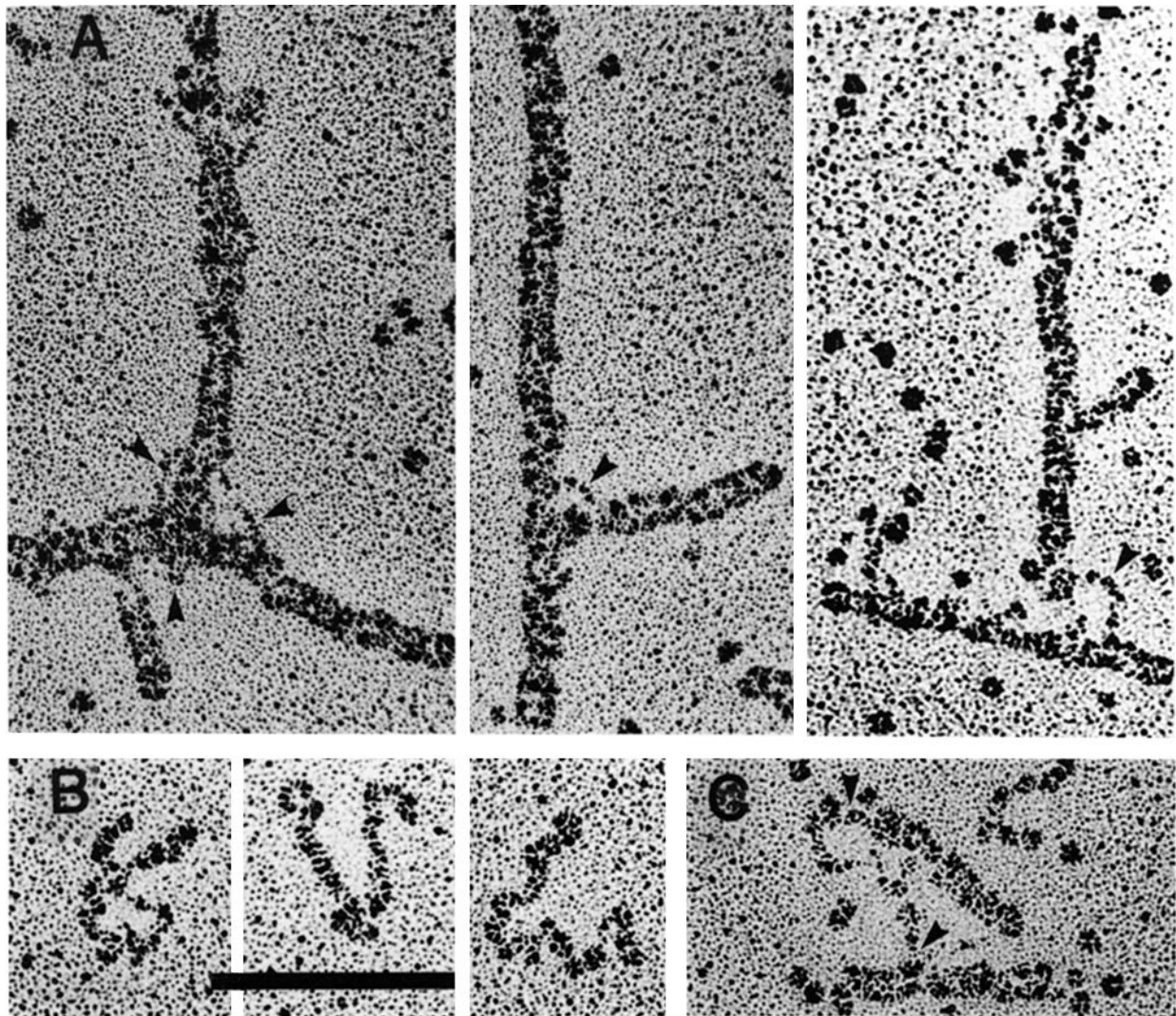


FIGURE 3 (A) Electron micrograph of actin copolymerized with actin-binding protein and rotary shadowed. G-actin, 1 mg/ml, and actin-binding protein, 1 mg/ml, in 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , and 2 mM Tris-HCl, pH 7.5, were mixed in a tube and made 2 mM with 1 M MgCl_2 and 100 mM with 3 M KCl. The tube was incubated for 10 min at 20°C and then diluted with 9 vol of 48% glycerol, sprayed onto mica, and rotary shadowed. The arrowheads point to strands with the same diameter as actin-binding protein molecules at actin filament branch points. (B) Electron micrographs of purified macrophage actin-binding protein dimers, 50 $\mu\text{g}/\text{ml}$, after spraying onto mica and rotary shadowing. (C) Electron micrograph demonstrating the direct cross-linking of preformed actin filaments by an actin-binding protein dimer. F-actin, 100 $\mu\text{g}/\text{ml}$, and actin-binding protein, 10 $\mu\text{g}/\text{ml}$, in 43% glycerol sprayed onto mica and rotary shadowed. The arrowheads point to regions of actin-binding protein F-actin attachment. Bars, 0.1 μm .

structure with shorter segments compared with the case of actin-binding protein added to preformed actin filaments (8). However, the L- or T-shaped branches alone would be insufficient for gelation of these actin solutions. Therefore, cross-linking of intersecting actin filaments by molecules of actin-binding protein in solution would be required to close the L- and T-shaped loops into a continuous network. X-shaped junctions provide an additional mechanism for closure of loops into a continuous network. Actin-binding protein dimers are large, highly flexible molecules, and we have proposed that their size and mobility permit them to bind to homologous sites in randomly overlapping actin filaments (8). The binding of actin molecules on actin-binding protein dimers can therefore be the same whether cross-linking occurs by a branching polymerization or by the tying together of preformed filaments.

The construction of a network by means of perpendicular

branching maximizes the efficiency of the individual cross-linking protein molecules by reducing the probability of intrafilament or redundant cross-links that do not contribute to enlargement of the network. Perpendicular cross-linking can also maintain the isotropy of the network by opposing the tendency for filaments to align side by side. It is interesting in this regard that the peripheral cytoplasm of highly motile cells such as macrophages and polymorphonuclear leukocytes lacks actin filament bundles and contains filament networks with short segments intersecting at more or less right angles (3, 30). The branching angles in cells could, of course, be altered by external forces or by tension generated by myosin.

The significance of branching polymerization for the quantitation of gelation in the attempt to isolate cross-linking factors from cells is worthy of comment. If actin is copolymerized with fractions of varying degrees of purity, nucleating effects of the

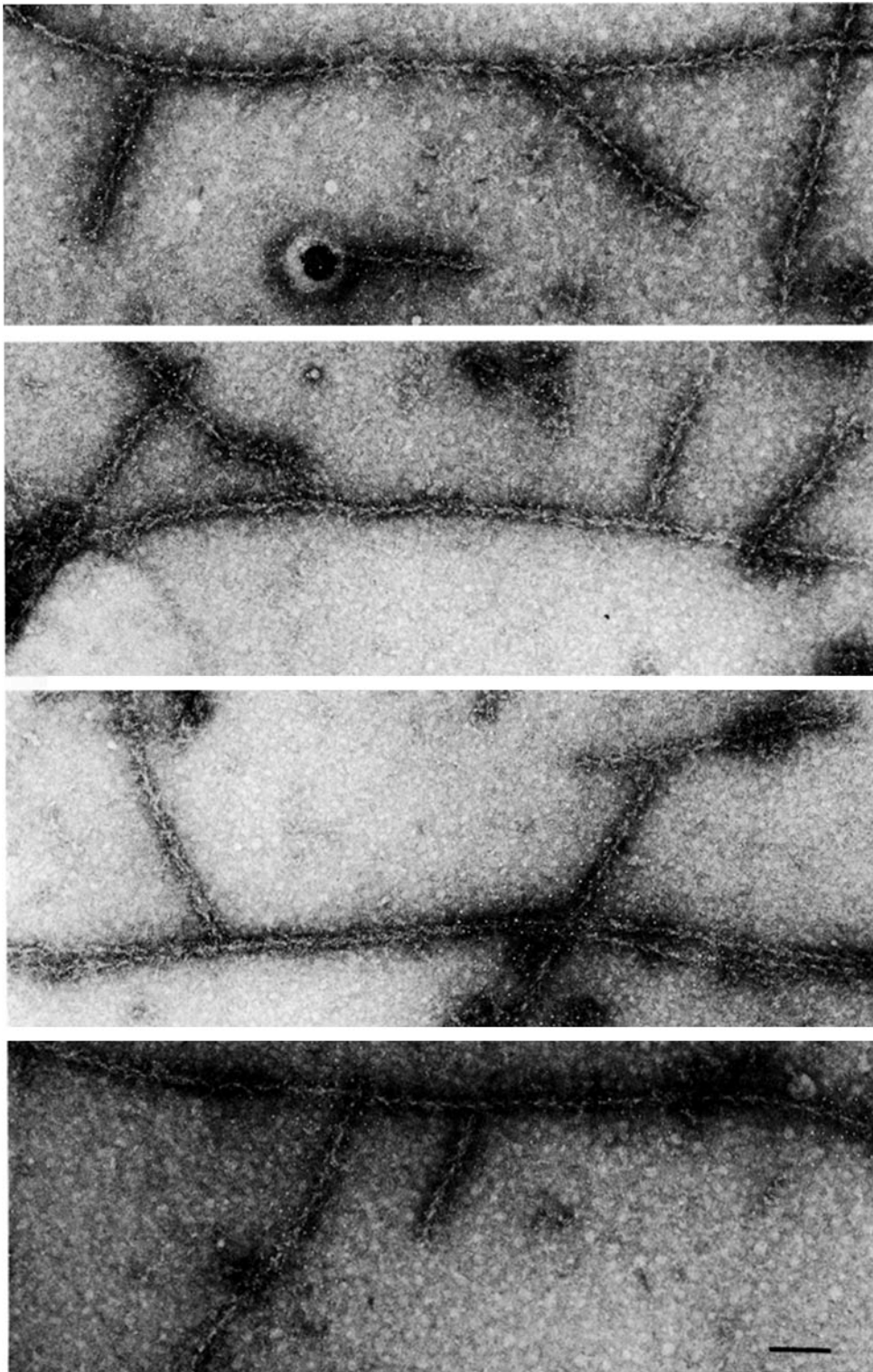


FIGURE 4 Electron micrographs showing the polarity of branching actin filaments. G-actin, 75 $\mu\text{g}/\text{ml}$, was polymerized in the presence of 25 $\mu\text{g}/\text{ml}$ of actin-binding protein on the surface of a grid. Both proteins were dialyzed against 0.5 mM ATP, 0.2 mM CaCl_2 , 0.5 mM 2-mercaptoethanol, and 2 mM Tris-HCl, pH 7.5, then mixed in a test tube. The polymerization was initiated by the addition of KCl to a final concentration of 0.1 M. One drop of the protein solution was immediately placed on a grid and incubated for 10 min at room temperature. The liquid was removed by touching the grid edge with filter paper, and one drop of heavy meromyosin, 0.2 mg/ml, in 0.1 M KCl, 10 mM imidazole-HCl, pH 7.5, was placed on the grid. The grids were incubated for 5 min more at room temperature, washed with 0.1 M KCl buffer, and then stained with 1% uranyl acetate. Of 200 branch points counted, 194 were found in which the filament growth was in the "preferred" direction (arrows pointing toward the branch points). Bar, 0.1 μm .

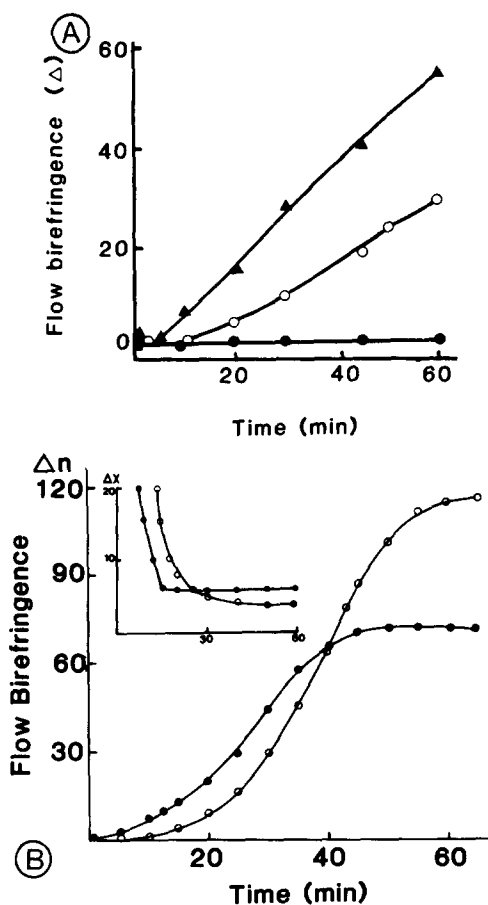


FIGURE 5 Effect of macrophage actin-binding protein on the onset of actin polymerization in 1 mM MgCl₂ (A) or 0.1 M KCl (B). (A) G-actin, in 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl₂, and 2 mM Tris-HCl, pH 7.5 was mixed in a test tube with actin-binding protein. 1 mM MgCl₂ was added to the actin solution (zero time), which was mixed and placed in the flow birefringence apparatus. The final concentration of actin was 0.25 mg/ml. Actin polymerized in the presence of 15 µg/ml (○) and 30 µg/ml (▲) of actin-binding protein and in the absence of actin-binding protein (●). All measurements were made at room temperature, using a velocity gradient of 100 s⁻¹. The final level of flow birefringence measured 19 h after the addition of MgCl₂ was 60° for actin alone, and 57° and 55° in the presence of 15 µg/ml and 30 µg/ml of actin-binding protein, respectively. (B) G-actin was mixed with actin-binding protein as in A. The solution was made 0.1 M with 3 M KCl (zero time), mixed, and placed in the flow birefringence apparatus. The final concentration of actin was 0.33 mg/ml. Actin polymerized in the presence of 30 µg/ml actin-binding protein (●) or in the absence of actin-binding protein (○). Inset: Effect of actin-binding protein on the extinction angle (ΔX) of actin copolymerized in the presence of 30 µg/ml actin-binding protein (●) or in the absence of actin-binding protein (○). The units of the vertical axes are degrees, and those of the horizontal axes (including the insert) are minutes.

cross-linking agent or of other factors in the medium on actin filament growth would be expected to alter markedly the length of the actin polymers at equilibrium. The critical concentration of cross-linking protein for incipient gelation of polymers is dependent on the polymer length distribution according to the expression $v_c = C/\bar{M}_w$, where v_c is the critical cross-linker concentration in moles/liter, \bar{M}_w is the weight average molecular weight of the polymers, and C is the concentration of

monomers in filaments, expressed in grams per liter (6). This relationship is valid for the cross-linking of actin by actin-binding protein (7, 35). In this study, \bar{M}_w of actin filaments polymerized in the presence of actin-binding protein was five-fold lower than that of actin polymerized alone. Therefore, effects of nucleation by a cross-linking agent can seriously complicate the interpretation of gelation activities.

The observed polarity of filaments at the branch points provides an excellent mechanism for permitting bipolar myosin filaments to generate force by engaging adjacent actin filaments emerging from a common branch point. This polarity also suggests a second mechanism for the movement of actin-containing cytoplasm in macrophages. The growth of new actin filaments from nuclei anchored on a preexisting actin and actin-binding protein lattice toward the membrane could generate a bulk expansion of cortical cytoplasm that could appear as a blunt pseudopod. This outward expansion requires that the growing lattice be buttressed by cellular structures sufficiently rigid to oppose inward expansion. Filaments growing toward the plasma membrane would have the polarity that has been described for membrane-associated actin, namely, that heavy meromyosin binds to these filaments to form arrowheads pointing away from the plasmalemma (2, 5, 21). This mechanism is a variation of the linear polymerization of actin proposed to explain the basis of the extension of acrosomal processes in certain marine sperm (29).

This study was supported by U. S. Public Health Service grants CA-09321, HL 19429, and HL 17411 and by a grant from the Council for Tobacco Research USA.

Received for publication 9 June 1980, and in revised form 11 August 1980.

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