Tracer Diffusion Through F-Actin: Effect of Filament Length and Cross-Linking

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ABSTRACT We have determined diffusion coefficients for small (50- to 70-nm diameter) fluorescein-thiocarbamoyl-labeled Ficoll tracers through F-actin as a function of filament length and cross-linking. fx45 was used to regulate filament length and avidin/biotinylated actin or ABP-280 was used to prepare cross-linked actin gels. We found that tracer diffusion was generally independent of filament length in agreement with theoretical predictions for diffusion through solutions of rods. However, in some experiments diffusion was slower through short ($\leq$1.0 μm) filaments, although this result was not consistently reproducible. Measured diffusion coefficients through unregulated F-actin and filaments of lengths >1.0 μm were more rapid than predicted by theory for tracer diffusion through rigid, random networks, which was consistent with some degree of actin bundling. Avidin-induced cross-linking of biotinylated F-actin did not affect diffusion through unregulated F-actin, but in cases where diffusion was slower through short filaments this cross-linking method resulted in enhanced tracer diffusion rates indistinguishable from unregulated F-actin. This finding, in conjunction with increased turbidity of 1.0-μm filaments upon avidin cross-linking, indicated that this cross-linking method induces F-actin bundling. By contrast, ABP-280 cross-linking retarded diffusion through unregulated F-actin and decreased turbidity. Tracer diffusion under these conditions was well approximated by the diffusion theory. Both cross-linking procedures resulted in gel formation as determined by falling ball viscometry. These results demonstrate that network microscopic geometry is dependent on the cross-linking method, although both methods markedly increase F-actin macroscopic viscosity.

INTRODUCTION

The structure and dynamic nature of the actin-based cytoskeleton in smooth- and nonmuscle cells is well established to be of prime importance for the control of cell shape, as well as for many cellular activities including motility, division, and various uptake and efflux processes. The specific structure of the cytoskeleton is regulated in a precise manner through the action of proteins known collectively as actin-binding proteins. These include proteins that regulate the length of actin filaments via capping and severing activities, cross-link the filaments into three-dimensional structures, and link the cytoskeleton to other membrane-bound and soluble cellular components (reviewed by Stossel et al., 1985). The mechanical properties of cytoplasm are due, at least in part, to cytoskeleton structure. However, many details of cytoplasmic architecture remain unclear.

One method for probing cytoskeleton structure is the measurement of the long-range translational diffusion of inert tracer particles through cytoplasm. This method is predicated on the fact that tracer diffusion is dependent solely on hydrodynamic interactions with intracellular components and, therefore, this parameter can reveal information about the higher-order structure of cytoplasm. Previous fluorescence recovery after photobleaching (FRAP) studies demonstrated that the normalized diffusion coefficient (D/D_o where D_o is the diffusion coefficient in an aqueous reference phase) of fluorescein-thiocarbamoyl (FTC)-labeled dextrans and Ficolls in Swiss 3T3 cells was steeply size dependent (Luby-Phelps et al., 1987). The fact that this size dependence was not found in protein solutions of concentrations comparable to that of cytoplasm indicated that it must arise from specific structural features imparted by the cytoskeleton (Hou et al., 1990).

A system of such complexity as cytoplasm is not conveniently described by theoretical treatments for tracer diffusion. To derive a model for cytoplasmic microarchitecture from diffusion data, it is necessary to construct an in vitro actin-based solution of defined structure that reproduces the tracer diffusion behavior found in cells. In an initial attempt along these lines, Hou et al. (1990) found that FTC-Ficoll tracer diffusion was size dependent in entangled F-actin networks, although the data did not conform quantitatively to the cellular data in that the decay of D/D_o as a function of tracer size was not as steep. In solutions of entangled F-actin and concentrated Ficoll or bovine serum albumin (BSA) it was found that D/D_o could be well approximated by multiplying the individual contributions from the two components. The effect of the background particles was to reduce D/D_o by a factor approximately equal to the bulk viscosity of the protein or Ficoll solution. From curve fitting and extrapolation it was predicted that a Ficoll volume fraction of 12.4% and an F-actin concentration of 37 mg/ml was necessary to reproduce the cellular data. This estimate of crowding in the background has since been confirmed (Kao et al., 1993) and corresponds to a soluble protein

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concentration of 110 mg/ml, which is consistent with the protein concentration in cells. However, the predicted actin concentration is significantly higher than that reported for cytoplasm. F-actin structure in cytoplasm is regulated by a variety of actin-binding proteins that sever, cap, and cross-link F-actin filaments, and this may produce a network geometry different from unregulated actin in vitro. Thus, to develop a more quantitative model for cytoplasm from diffusion data, it is necessary to determine the specific contribution to tracer diffusion of the complex array of factors relating to actin structure.

In the present study, we determined the effect of actin filament length and cross-linking on diffusion of FTC-labeled Ficoll tracers. We found that diffusion rates were generally independent of filament length, and cross-linking with ABP-280 resulted in a significant decrease in tracer diffusion. These results suggest that the predicted contribution of F-actin networks on cellular diffusion data can be reasonably approximated in vitro under certain conditions. In contrast to ABP-280 cross-linking, avidin-induced cross-linking of biotinylated F-actin under conditions that resulted in gel formation was not sufficient to retard tracer diffusion, and actually increased diffusion under certain conditions in a similar manner to that observed in a previous study of actin-filamin mixtures (Hou et al., 1990). These results are discussed in terms of in vitro modeling of cytoplasm structure.

MATERIALS AND METHODS

Protein purification

Actin was purified from rabbit skeletal muscle according to Spudich and Watt (1971). For some experiments, actin was purified further by gel exclusion chromatography using a Sephacryl S-300 (95 × 17 cm) column. Actin was dialyzed into Buffer G (2 mM Tris, pH 8.0, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.3 mM NaN_3) and stored as aliquots under liquid nitrogen. Before use, monomeric actin (G-actin) was clarified using an airfuge (120,000 × g for 20 min). Avidin was purchased from Molecular Probes (Eugene, OR) as a lyophilized powder and dissolved in Buffer G for use. fx45, (the actin-binding domain of gelsolin) was a gift from Dr. Helen Yin. Macrophage ABP-280 was a gift from Dr. John Hartwig.

Labeling and purification of fluorescein-Ficoll tracer

F400 (Pharmacia Fine Chemicals, Piscataway, NJ) was activated and labeled with fluorescein-5-isothiocyanate according to the procedure described by Luby-Phelps (1988). The FTC-Ficoll mixture was size fractionated on a 5 × 100 cm column of Sepharose CL-6B (Pharmacia Fine Chemicals) in 10 mM Tris, 50 mM KCl, 0.02% NaN_3, pH 8.0 at 20°C. The mean hydrodynamic radius of the tracer fraction used was determined using FRAP (see below) to measure the diffusion coefficient in aqueous buffer (D_o). The radius was then calculated from the Stokes-Einstein relationship. The tracer radii for specific experiments are given in the Results.

Biotinylation of actin

Iodoacetyl-N'-biotinhexenediamine (Pierce Chemical Co., Rockford, IL) was used to biotinylate actin essentially as given by Wachsstock et al. (1994). Lyophilized actin was dialyzed into 25 mM imidazole, pH 7.5, 0.3 mM ATP, 0.3 mM NaN_3 at a concentration of 24 mM (1 mg/ml). Actin was polymerized by bringing the solution to 0.1 M KCl, 2 mM $MgCl_2$ and incubating at room temperature for 30 min. A sevenfold molar excess of iodoacetylbiotin (40 mM in dimethylformamide) was added to the F-actin sample and the solution was rotated slowly overnight at 4°C in the dark. The polymerized biotinylated actin was then pelleted (2 h at 200 × g), dilayed into Buffer G and then passed over a G-25 Sephadex column to remove residual free biotin. The extent of biotinylation was measured by the displacement of 2-(4′-hydroxyazobenzene)benzoic acid from avidin using a kit from Pierce Chemical Co. (Rockford, IL). The actin used in experiments given here was 28% biotinylated (e.g., 28% of actin monomers were biotinylated given that there is only one reactive cysteine per actin monomer) and diluted to the desired final percentage of biotinylation by mixing with unlabeled actin. Biotinylated actin was stored as given above and also airfuge-clarified before use.

Falling ball assay

Determination of the shear viscosity of F-actin solutions was carried out using falling ball viscometery (MacLean-Fletcher and Pollard, 1980). All measurements were carried out at 1 mg/ml F-actin. Actin, biotinylated actin, fx45, and Buffer G were combined and actin was polymerized by adding KCl and $MgCl_2$ to final concentrations of 0.1 M and 2 mM, respectively. The samples were then immediately drawn into 100 μl capillary tubes and incubated for 2 h at room temperature. The assay was then carried out by dropping stainless steel balls (0.025-inch diameter; The Micro Ball Company, Peterborough, NH) on the samples and measuring the falling time. For experiments in which the effect of cross-linking was evaluated, samples were prepared according to either a pre- or copolymerization protocol. For the former, samples were prepared and then polymerized by incubating for 30 min at room temperature after addition of KCl and $MgCl_2$. Cross-linker (avidin in buffer G or ABP-280 in TBS) was then added and the solution was drawn into the capillary tubes. For the copolymerization procedure, cross-linker was added before initiating polymerization and samples were then prepared as given above.

Sample preparation for FRAP experiments

Samples for FRAP experiments were prepared essentially as described above for the falling ball assay in a total volume of 25 μl with a final concentration of FTC-Ficoll <0.1%. Both the pre- and copolymerization procedures described above were used. Samples were drawn into microslides (either 0.05- or 0.10-mm path length; Vitro dynamics, Rockaway, NJ) that were precoated with BSA to prevent nonspecific adsorption of the tracer. These were then placed on 3 × 1-inch microscope slides (Becton DickInson Labware, Franklin Lakes, NJ) and sealed with Pro-texx sealant (Lerner Laboratories, Pittsburgh, PA). The samples were incubated overnight in the dark at room temperature before FRAP measurements.

FRAP measurements

FRAP was performed at room temperature as described previously (Tansey et al., 1994). Photobleaching times ranged from 1 to 20 ms, and recoveries of ~10 half-lives were monitored. Up to 10 measurements were made for each sample. Recovery curves were analyzed according to the approximation given by Yguerabide et al. (1982). All results shown represent averages obtained from at least duplicate independent experiments.

Light scattering

Turbidity measurments were carried out on a Pharmacia Ultrospec III UV/vis spectrophotometer. Samples were prepared by methods given above. Specific details are given in Results.

Fluorescence microscopy of actin filaments

For visualization of actin filaments by fluorescence microscopy, rhodamine-phalloidin staining was used (Kron et al., 1991). Briefly, 50 μl of 3.3 μM rhodamine phalloidin (Molecular Probes, Eugene, OR) were dried down from methanol, after which actin samples were added to yield a final actin concentration of 3 mg/ml (dye/actin = 0.097). The samples were incubated at room temperature overnight and images were obtained at 100× magnification, using a cooled CCD camera (Photometrics, Tucson, AZ) as previously described (Luby-Phelps et al., 1995).

RESULTS

Experimental system

For studies of how filament length affects tracer diffusion, we used fx45, the actin-binding domain of gelsolin that caps and severs actin filaments in a Ca^{2+}-independent manner (Yu et al., 1991), to regulate F-actin length. Filament lengths were estimated based on the assumption that a given actin filament is capped by one fx45; thus, length is given by the molar ratio of fx45:actin in conjunction with the known length per actin monomer of 2.7 nm (Janmey et al., 1986; e.g., fx45:actin of 1:370 yields an average filament length of 1 μm). The effect of actin cross-linking on diffusion was determined by using two cross-linking systems: avidin/biotinylated actin and ABP-280/actin. These methods are both known to yield stable cross-linked actin gels (Brotschi et al., 1978; Janmey et al., 1990; Wachsstock et al., 1994). ABP-280 is a physiological protein cross-linker reported to induce formation of isotropic actin networks (Hartwig et al., 1980; Niederman et al., 1983). All experiments were carried out with a FTC-Ficoll tracer of either 50 or 70 nm in diameter. These tracers are large enough so that their diffusion is hindered significantly at moderate actin concentrations (1–3 mg/ml, Hou et al., 1990) but not so large that diffusion becomes too slow to measure accurately (see Qian et al., 1992).

To demonstrate that interaction of the tracer with avidin would not contribute to the results in the avidin/biotinylated actin system, we carried out the following control experiment. Avidin was immobilized by coating microslides with biotinylated BSA followed by addition of avidin. The mobility of rhodamine-avidin was measured by FRAP to confirm avidin immobilization. Tracer diffusion was measured in microslides coated with immobilized, unlabeled avidin or with BSA alone. We found that the diffusion of the tracer was identical in the presence and absence of avidin, demonstrating that the tracer did not interact with avidin.

Tracer diffusion is generally independent of actin filament length

The length of F-actin filaments likely influences the geometry of actin networks. However, the dependence of small tracer diffusion on filament length has not been investigated systematically. We found that, in general, normalized tracer diffusion (D/D_o, where D_o is the diffusion coefficient in F-buffer) was independent of filament length over a wide range of lengths (Fig. 1 *A*). However, we sometimes observed that diffusion was as much as 15% slower for the shorter (1 μm and below) filaments (Fig. 1 *B*) although this decrease was not consistently reproducible. This result was not dependent on whether actin was gel filtered, and did not appear dependent on variations in sample handling methods. Furthermore, this difference in length dependence did not correlate with actin concentration, fractional biotinylation or a particular batch of fx45 or avidin. However, within a given set of samples prepared in parallel under the same

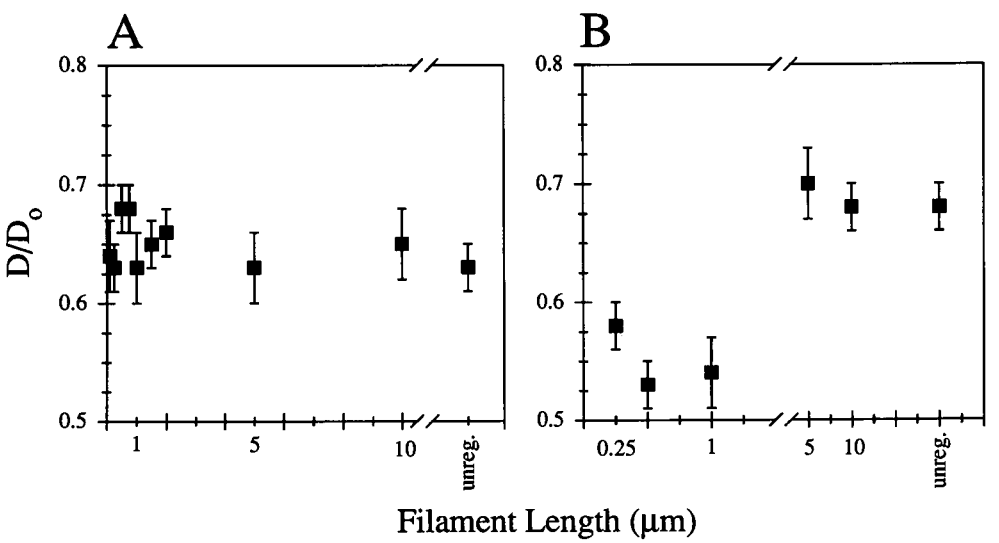

FIGURE 1 Dependence of tracer diffusion on filament length. (*A*) D/D_o for a 70-nm Ficoll tracer as a function of F-actin filament length when no length dependence was observed. F-actin concentration was 3 mg/ml. (*B*) D/D_o for a 70-nm Ficoll tracer as a function of F-actin filament length showing hindered diffusion for actin filaments of ≤1 μm. F-actin concentration was 3 mg/ml. Filament length was regulated by varying the actin/fx45 ratio.

conditions, either the length-independence or the slower diffusion for the shorter filaments was very reproducible. In contrast to these results, the shear viscosity of F-actin as determined by the falling ball assay was quite sensitive to filament length (shown in Fig. 2 for 1 mg/ml F-actin) as shown by previous investigators (Janmey et al., 1988; Zaner and Hartwig, 1988). We also observed that normalized tracer diffusion depended markedly on F-actin concentration as demonstrated previously (Hou et al., 1990).

Avidin-induced cross-linking of biotinylated actin enhances tracer diffusion

If cross-linking of F-actin results in formation of networks with a smaller average pore size than that of uncrosslinked actin, it is anticipated that this will result in retardation of tracer diffusion. As a first attempt along these lines, we elected to measure tracer diffusion through avidin/biotinylated-actin cross-linked F-actin filaments. This cross-linking method has been shown to result in F-actin gels that have rheological properties resembling a covalent network and similar to those of ABP280/actin networks (Janmey et al., 1990; Wachsstock et al., 1994).

In the absence of fx45 and when D/D_0 was length-independent in the presence of fx45, avidin-induced cross-linking of F-actin filaments had no significant effect on tracer diffusion (results shown for 3 mg/ml F-actin in Fig. 3 *A*). The results shown were obtained with avidin added after polymerization of actin (prepolymerization protocol); identical results were obtained if avidin was added before polymerization (copolymerization protocol, not shown). D/D_0 values were insensitive to avidin cross-linking up to 8 mol % biotinylated actin and 6 mg/ml F-actin (not shown). We also found that diffusion was unaffected if double the saturating amount of avidin was added, or when 1/2× molar-free biotin was included with the avidin to decrease the average number of free-binding sites on avidin from four to two (not shown). These results demonstrate that avidin/biotinylated actin cross-linking did not influence network geometry in a manner that affected diffusion of a tracer molecule of this size range. Nevertheless, cross-linking did result in gel formation as shown by the falling ball assay for 2% biotinylated actin at 1 mg/ml. The ball remained stationary at the top of the capillary tube as expected for a cross-linked gel. Inclusion of biotinylated actin (from 2 to 8 mol %) in the absence of avidin had no effect on the falling times for F-actin (not shown).

In contrast, whenever D/D_0 was observed to be slower for the shorter filaments, avidin-induced cross-linking of these filaments tended to increase tracer diffusion coefficients and yielded D/D_0 values similar to those observed for the long, unregulated filaments (representative data are shown in Fig. 3 *B* for 1-μm filaments). The magnitude of the increase in diffusion coefficient was independent of fractional biotinylation over the range examined. In all cases, avidin-induced cross-linking of 1.0, 0.50, and 0.25 μm filaments with 2% biotinylated actin did result in gel formation as shown by the falling ball assay. Gel formation was not evident for 0.10-μm filaments at 2% biotinylation, but did occur with 10% biotinylation.

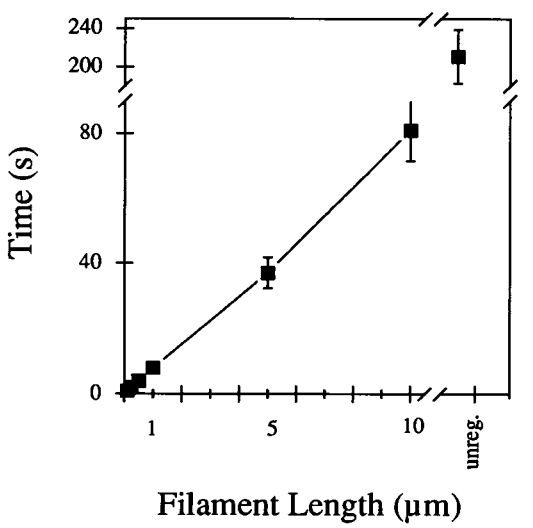

FIGURE 2 Falling ball viscometry as a function of actin filament length. The *y* axis is the time required for balls to settle to the bottom of 100-μl capillary tubes containing 1 mg/ml F-actin (see Materials and Methods for details). Filament length was regulated by varying the actin/fx45 ratio. Results represent averages from two independent experiments with each sample prepared in duplicate. In contrast to tracer diffusion, bulk viscosity is proportional to filament length.

Cross-linking of actin by ABP-280 retards tracer diffusion

Unlike many other actin-binding proteins, ABP-280 (nonmuscle filamin) is reported to cross-link F-actin into isotropic networks rather than bundles (Hartwig et al., 1980; Niederman et al., 1983). We found that 1 mol % ABP-280 at a physiological actin concentration (4 mg/ml) significantly decreased diffusion of a 50-nm diameter tracer (Fig. 4 *A*) through unregulated F-actin. This result did not depend on whether ABP-280 was added before polymerization (copolymerization protocol) or after polymerization (prepolymerization protocol). Very similar results were obtained for 5 mg/ml actin with 0.75 mol % ABP-280 (not shown). For short (0.5-μm filaments) ABP-280 cross-linking also tended to reduce diffusion although the difference was not significant relative to uncrosslinked 0.5-μm filaments ($p > 0.05$; Fig. 4 *B*). The gels formed in the presence of ABP-280 by either protocol were indistinguishable by falling ball viscometry both for unregulated and 0.5-μm filaments. We also found that 0.2% ABP-280 was sufficient to substantially increase falling times for unregulated F-actin (approximately sevenfold), in agreement with the previous obser-

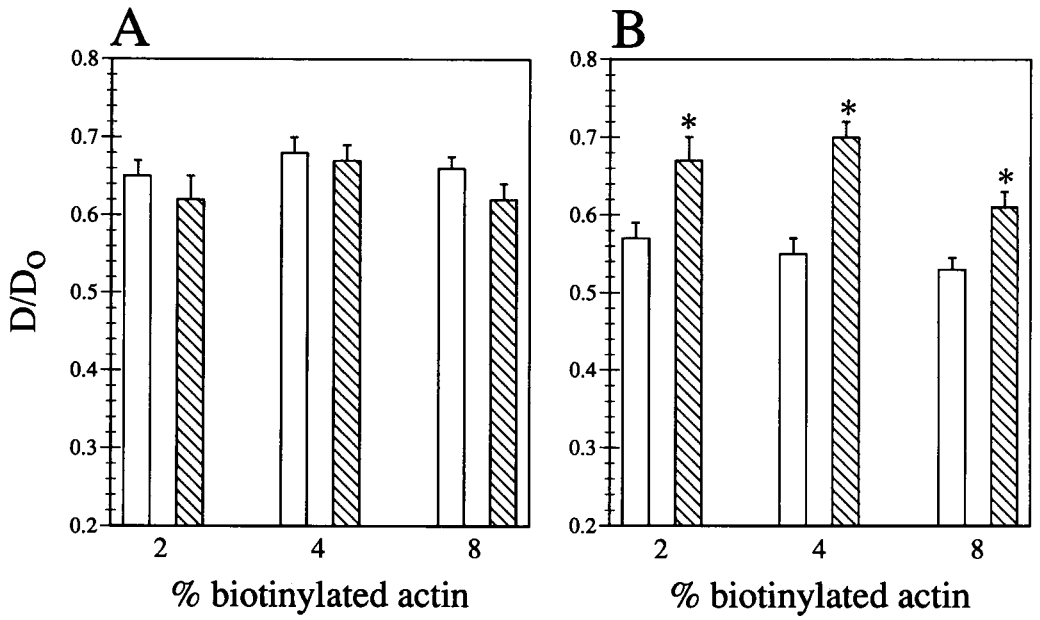

FIGURE 3 Diffusion of 70-nm Ficoll tracer through F-actin as a function of avidin cross-linking for (*A*) unregulated F-actin filaments and (*B*) 1-μm filaments when length-dependence was observed. The unshaded bars are without avidin and the cross-hatched bars are with avidin at a molar avidin/biotin ratio of 0.3 (i.e., saturating avidin). Samples were prepared using the prepolymerization protocol. F-actin concentration is 3 mg/ml. The * denotes a statistically significant difference in D/D_o ($p < 0.05$). Avidin cross-linking of the 1-μm filaments increases diffusion rates to the level of diffusion through unregulated filaments (or short filaments when length-independent).

vation that this cross-linking density coincides with the onset of gel formation by this protein (Janmey et al., 1990).

Avidin-biotinylated actin and ABP-280-actin form cross-linked gels with different geometries

One explanation for the slower diffusion of tracer in shorter filaments is that the longer filaments exhibit some degree of bundling. The fact that avidin-induced cross-linking abolished the difference in diffusion rates through long and short filaments when this phenomenon occurred suggests that this cross-linking method induces bundling of F-actin (see Discussion). To examine this possibility, we carried out steady-state turbidity measurements. 1-μm filaments exhibited a significantly lower OD_{600} value than the unregulated filaments in the absence of avidin (Fig. 5 *A*). Avidin-induced

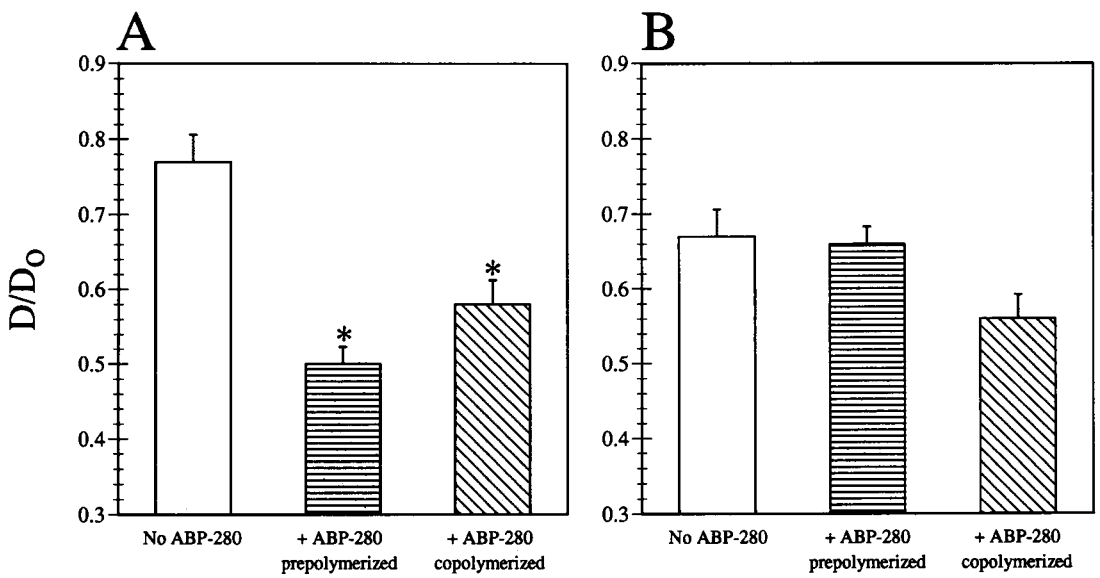

FIGURE 4 Diffusion of 50-nm Ficoll tracer through F-actin as a function of ABP-280 cross-linking for (*A*) unregulated F-actin filaments and (*B*) 0.5-μm filaments. F-actin concentration is 4 mg/ml. The * denotes a statistically significant difference in D/D_o ($p < 0.05$). ABP-280 cross-linking hinders tracer diffusion through unregulated F-actin but has no significant effect on diffusion through 0.5-μm filaments.

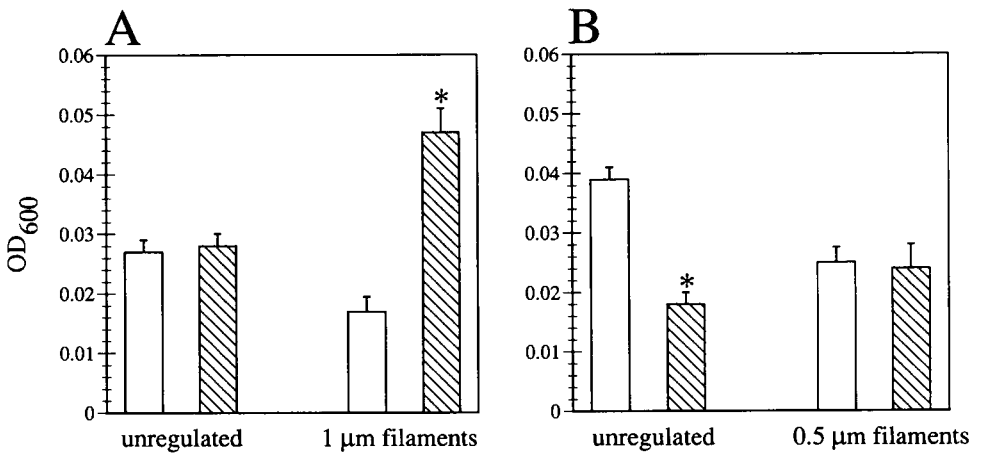

FIGURE 5 Turbidity of F-actin as a function of avidin and ABP-280 cross-linking. (*A*) The unshaded bars are without avidin and the cross-hatched bars are with avidin at a molar avidin/biotin ratio of 0.3. F-actin concentration is 3 mg/ml with 2 mol % biotinylated actin. Avidin cross-linking of the 1-μm filaments results increases turbidity. (*B*) The unshaded bars are without ABP-280 and the cross-hatched bars are with ABP-280 at a molar ABP-280/actin ratio of 0.01. The * denotes a statistically significant difference in OD_{600} ($p < 0.05$). F-actin concentration is 4 mg/ml. ABP-280 cross-linking of unregulated filaments decreases turbidity. Results represent averages from two independent experiments.

cross-linking of the unregulated filaments at 4% biotinylated actin did not affect the OD value, consistent with the lack of effect on tracer diffusion. However, cross-linking of the 1-μm filaments under these conditions resulted in a nearly fourfold increase in the OD_{600} (0.047 as opposed to 0.017), which supports the hypothesis of F-actin bundling.

In contrast to the turbidity results with avidin-induced cross-linking, ABP-280 cross-linking significantly decreased the OD_{600} of unregulated F-actin, but had no significant effect on short filaments (Fig. 5 *B*). These findings, in conjunction with the differing effects on tracer diffusion, indicate that actin gels of distinct geometries result from these two cross-linking methods and suggest that ABP-280 inhibits bundling.

We also carried out fluorescence microscopy with rhodamine-phalloidin staining of actin filaments in an attempt to visualize bundles. We were unable to detect any difference in the morphology of unregulated and short (1 μm) filaments (not shown). Given that the inherent resolution limit of this method is ~200 nm, this procedure cannot be used to distinguish individual filaments from small bundles under optimal conditions. The relatively high actin concentration (3 mg/ml) used in our studies also likely limited our working resolution. We were also unable to detect any difference in morphology between long and short filaments by phase-contrast microscopy.

DISCUSSION

To examine in vitro models for cytoplasmic architecture by tracer diffusion, it is necessary to understand in detail the effect of actin filament organization on the kinetics of the diffusion process. Our results show that cross-linking of F-actin significantly influences diffusion rates, and that the specific effect of cross-linking on diffusion varies considerably depending on experimental conditions and choice of cross-linker. We will discuss the results with the aim of correlating observed diffusion rates with actin structural organization and subsequently interpret our findings in terms of theoretical predictions for tracer diffusion through rod-like networks.

The fact that tracer diffusion is generally independent of filament length is consistent with theoretical predictions that diffusion should depend on net filament length per unit volume, which may also be taken as filament surface area (Ogston et al., 1973; Han and Herzfeld, 1993). The observation that relatively short filaments have some tendency toward reducing tracer diffusion suggests that the longer filaments may exhibit a greater degree of bundling. This is consistent with our turbidity and fluorescence microscopy results. Lateral association of filaments decreases the average net concentration of obstacles and is thus predicted to increase tracer diffusion rates (see below). The tendency for rod-like structures to adopt parallel aligned structures is well established from both theoretical considerations (Madden and Herzfeld, 1993) and empirical observations (see below). At sufficiently high concentrations, the rotational freedom gained from alignment of filaments more than compensates for the loss of translational freedom. Spontaneous conversion of F-actin from isotropic networks into ordered liquid crystalline arrangements is well known, and the actin concentrations of 3–4 mg/ml used in the present study is within the range of values previously reported for F-actin alignment (Cortese and Frieden, 1988; Furukawa et al., 1993; Kerst et al., 1990). Futhermore, the tendency of rods to aggregate is proportional to rod length, because of the greater tendency of longer rods to overlap, thus inducing the entropically driven conversion to parallel rod alignment. The dependence of F-actin bundling on filament length has been described from a theoretical standpoint (Madden and

Herzfeld, 1994) and has been documented in a number of studies (Cortese and Frieden, 1988; Furukawa et al., 1993; Suzuki et al., 1991). In interpreting our tracer diffusion results in terms of actin morphology, we were mindful of the fact that these relatively short filaments have diffusion coefficients of very similar magnitudes as that of the tracer molecule (e.g., 3.3×10^{-8} cm^2/s for the tracer through 1-μm filaments as opposed to $\sim 2 \times 10^{-8}$ cm^2/s for the filaments (Janmey et al., 1986)). Mobility of the obstacles is predicted to increase diffusion of tracer molecules through networks, although quantitative estimates of this effect are problematical (Muhr and Blanshard, 1982). Nonetheless, the fact that we sometimes see reduced tracer diffusion through short filaments provides strong evidence that the short filaments have a reduced bundling tendency relative to long filaments.

We were unable to identify why hindered diffusion through the short filaments (≤ 1 μm) was observed only in certain experiments. As discussed above, no obvious difference in material stocks or sample preparation methods could account for this observation. We think that some feature of sample handling that was beyond our control most likely affected whether or not the short filaments formed bundles in a given experiment. It is well known that the macroscopic properties of actin can depend on sample handling (see e.g., Furukawa et al., 1993), and we may well have had different levels of shearing, slightly different temperatures, etc., on different days.

The finding that avidin-induced cross-linking of unregulated biotinylated F-actin had no effect on tracer diffusion suggests that this cross-linking method does not have a marked influence on microscopic actin organization under these conditions. This conclusion is supported by the fact that sample turbidity was also insensitive to avidin cross-linking. However, the falling ball assay clearly demonstrated that this cross-linking procedure results in gel formation, in agreement with previous reports (Janmey et al., 1990; Wachsstock et al., 1994). Thus, our results suggest that avidin cross-linking of unregulated biotinylated F-actin results in gels with no change in average pore size or that the change was not sufficient to be detected by probes in the 50- to 70-nm diameter range and bundling relative to uncrosslinked F-actin. Collectively, these findings underscore the very different properties of actin networks in terms of the "macroviscosity" detected by falling ball viscometry and the "microviscosity" sensed by tracer diffusion.

The significant increase in tracer diffusion sometimes evident upon avidin-induced cross-linking of the short filaments (≤ 1 μm) strongly suggests that these filaments undergo a transition from noninteracting (or weakly interacting) polymers to a bundled structure. This interpretation was confirmed by the observed increase in turbidity upon avidin cross-linking. An increase in tracer diffusion concomitant with bundling of F-actin by filamin was observed previously (Hou et al., 1990). Thus, avidin-cross-linked biotinylated actin more closely resembles filamin/actin than ABP-280/actin in terms of network geometry.

The fact that ABP-280 cross-linking decreased tracer diffusion through unregulated F-actin strongly suggests that isotropic networks as opposed to bundles are formed in this case. The conclusion that ABP-280 cross-linking results in formation of isotropic networks is consistent with findings from previous electron microscopy studies of ABP-actin gels. Hartwig and Stossel (1981) found that addition of ABP-280 to prepolymerized actin below ~ 1 mol % did not affect actin organization, although subtle changes were observed at mole ratios of 2% or 5%. However, polymerization of actin in the presence of ABP-280 clearly promotes bipolar, perpendicular branching of actin filaments even at molar ratios as high as 4% (Hartwig et al., 1980; Niederman et al., 1983). This network arrangement closely resembles the peripheral cytoskeleton of motile cells. The fact that we saw a similar decrease in tracer diffusion for both pre- and copolymerization suggests that ABP-280 altered actin organization independently of sample preparation method under the conditions employed in the current study.

It is instructive to compare our results with theoretical predictions for diffusion of small tracer molecules through networks. Most theories predict a square-root dependence of diffusion on filament length per unit volume. For convenience, we chose to analyze our results in terms of the Ogston treatment (Ogston et al., 1973), which is a detailed theory for the diffusion of particles through solutions of rigid, immobile (or very slowly moving), rod-like polymers. This theory holds that

$$D/D_o = \exp[-(\pi L)^{1/2}(r + \rho)]$$

where L is the length of the polymer chain per unit volume, r is the hydrodynamic radius of the diffusing particle, and ρ is the polymer radius. According to this treatment, because diffusion depends on net filament length per volume, D/D_o is strongly dependent on F-actin concentration but is independent of average filament length at a given concentration. Lateral association among filaments would act to effectively decrease the L value leading to a corresponding increase in predicted diffusion rates, unless the resultant increase in polymer radius significantly increases the $r + \rho$ term. Along these lines, Han and Herzfeld (1993) in their theoretical treatment of diffusion in crowded solutions predicted that diffusion rates through an array of rods should depend on net rod surface area, and thus be independent of average rod length at a given concentration. However, given that tracer diffusion has not been examined previously under conditions in which both filament length and cross-linking have been systematically varied, the applicability of these models to diffusion through F-actin is not known. We will use the Ogston theory as a framework for interpretation of our results and consider implications for F-actin organization under the various conditions examined.

As an example, we will consider the results at 4 mg/ml F-actin with a 50-nm diameter tracer. Under these conditions, $L = 155$ μm/μm^3, which yields a predicted D/D_o value of 0.46 with the assumption of an F-actin diameter of

10 nm (Pollard and Cooper, 1986). The fact that our measured D/D_0 value (0.77) for unregulated F-actin is significantly higher than that predicted is consistent with the explanation that these filaments are not monodisperse but are likely aggregated to some degree. In view of the limitations inherent in modeling unregulated F-actin as a rigid rod because of complications arising from internal motion of actin chains (despite a significant persistence length of ~17 μm (Gittes et al., 1993)) and length polydispersity, we must be cautious in drawing quantitative conclusions regarding unregulated actin organization from diffusion data. Nonetheless, our findings support the hypothesis that F-actin has the capacity to form aligned domains in this concentration range.

Our results with unregulated actin are essentially identical to those observed in a previous study that employed equivalent procedures for actin purification and sample preparation. Hou et al. (1990) observed *D/Do* values of 0.68 and 0.42 at 3 and 12 mg/ml actin, respectively, for a 60-nm diameter Ficoll tracer compared with theoretically predicted values of 0.51 and 0.26. Qian et al. (1992) obtained diffusion values for somewhat larger fluorescent bead tracers through 1 mg/ml F-actin that were generally in closer agreement with the Ogston theory than those obtained in this study and in Hou et al. (e.g., observed and predicted values of 0.71 and 0.61, respectively, for a 90-nm tracer and 0.17 and 0.21, respectively, for a 280-nm tracer).

Interestingly, our observed D/D_0 value for unregulated actin cross-linked with 1 mol % ABP-280 (average value of 0.54) is very much in line with the value of 0.46 predicted by the Ogston treatment. It therefore seems that this situation most closely approximates diffusion through a rigid, random network as predicted by this model. This result strongly supports our conclusion of significant bundling among unregulated (and avidin-cross-linked) actin filaments. The fact that no significant effect of ABP-280 cross-linking was observed for short 0.5-μm filaments may reflect that these filaments are less strongly bundled initially (as shown by the turbidity results), and thus the "debundling" effect of ABP-280 is less pronounced. It is also possible that meshworks of larger average pore size are formed for the smaller filaments, which have less tendency to overlap.

The treatment of Ogston does not explicitly address the case of a cross-linked meshwork in which cross-linking is expected to decrease the average pore size of the meshwork. The effect of cross-linking on diffusion through meshworks can be modeled according to:

$$D/D_0 = \exp(-\beta(d/\xi)^{\partial}),$$

where β is ~1, d is probe diameter, ξ is average mesh size, and ∂ is an exponent, which is 2.5 for cross-linked networks and 2.0 for noncrosslinked networks (Schmidt et al., 1989). Thus, tracer diffusion is inversely proportional to meshwork diameter, and when tracer diameter becomes large with respect to the mesh size, cross-linking is predicted to substantially retard diffusion. Based on this treatment, an average mesh size of 100 nm can be calculated from our data on ABP-280 cross-linking of unregulated F-actin. Because the diameter of the tracer used in this study is smaller than the average mesh size, cross-linking is not expected to decrease tracer diffusion rates. This likely accounts for the fact that cross-linking did not yield D/D_0 values below that predicted by the Ogston theory for rigid, random networks. Thus, the hindered tracer diffusion obtained when actin is polymerized in the presence of ABP-280 can be explained by the fact that networks as opposed to bundles are formed.

Our finding that tracer diffusion is significantly retarded by ABP-280 cross-linking under certain conditions demonstrates that diffusion rates more akin to those for fluorescent tracers in the cytoplasm of living cells are possible at physiological actin concentrations. The D/D_0 value we obtained with 1% ABP-280 cross-linking at 4 mg/ml F-actin is very similar to that observed for 12 mg/ml actin in the absence of cross-linker (Hou et al., 1990). Generalizing this fourfold decrease in the diffusion coeffficient of the tracer to higher actin concentrations, we speculate that for unregulated filaments a cross-link density of 1% would reduce the F-actin concentration required to obtain a quantitative fit of the cytoplasmic data to our previous model from 37 mg/ml to ~9 mg/ml. This actin concentration and ABP-280:actin ratio approach the physiological range. We were unfortunately unable to attempt experiments with higher actin concentrations while maintaining physiological ABP-280: actin ratios because of technical limitations inherent in concentrating ABP-280. Additional factors that may increase the volume fraction occupied by the network and further reduce the concentration of actin filaments required include the likelihood that many cellular components bind to actin (e.g., Bassell et al., 1994; Condeelis, 1995; O'Reilly and Clarke, 1993) thereby increasing the effective filament diameter.

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