Phalloidin Binding and Rheological Differences among Actin Isoforms[†]

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ABSTRACT: Actin is a highly conserved protein in eukaryotes, yet different isoforms of this protein can be found within the same cell. To begin to explore whether isoactin sequence diversity leads to functional differences in actin filaments, we have examined the phalloidin binding kinetics and the bulk rheologic properties of purified actin isoforms from a variety of eukaryotic sources. We observe differences in the phalloidin association kinetics between muscle α- and cytoplasmic actins. Phalloidin dissociates from all mammalian actin isoforms tested at the same slow rate, while dissociation from yeast actin is 1 order of magnitude more rapid. The actin isoforms form viscoelastic gels to varying degrees with skeletal muscle α-actin gels being the most elastic, smooth muscle α- and γ-actins being less elastic, and β-actin not forming elastic structures under our experimental conditions. The sequence variation among isoforms is discussed in light of these biophysical and biochemical differences.

Actins are a ubiquitous family of eukaryotic cytoplasmic proteins. These proteins are highly conserved, differing in sequence identity by less than 40% between fungi and humans [see Herman (1993) and Sheterline et al. (1995) for review]. Yet even within vertebrates, there are six actin isoforms which differ in their isoelectric point and site of expression. These isoforms are grouped into α, β, and γ forms on the basis of their p*I* (Garrels & Gibson, 1976) and can be further classified by the amino acid sequence and place of expression into six subtypes: two in striated muscle (α-skeletal and α-cardiac actins), two in smooth muscle (α-vascular and γ-enteric actins), and two in nonmuscle cells (β- and γ-cytoplasmic actins) [see Vanderkerckhove and Weber (1978) and Rubenstein (1990) for review].

Many cell types express more than one isotype of actin, and in many cells, these actins are segregated to different compartments of the cytoplasm (DeNofrio et al., 1989; Hoock et al., 1991; North et al., 1994; Shuster & Herman, 1995; Yao et al., 1995). This segregation occurs both at the site of isoactin message expression (Hill & Gunning, 1993; Hoock et al., 1991; Taneja & Singer, 1990; Yeh & Svoboda, 1994) and at the localization of individual proteins (DeNofrio et al., 1989; Hoock et al., 1991). β-Actin has been shown to concentrate in motile, noncontractile areas of cells (DeNofrio et al., 1989; Hill & Gunning, 1993; Hoock et al., 1991), while muscle actins are found predominantly in stress fibers or sarcomeric-like structures (Gunning et al., 1984). While cytoplasmic and muscle actins differ by less than 22 residues in 374 amino acids, cytoplasmic actins do not seem to be able to complement muscle actins in the formation of contractile structures in muscle and myocytes (Schevov et al., 1992; Schoenenberger et al., 1995; von Arx et al., 1995).

There have been a limited number of reports of actin binding proteins preferring one isoform over another. This may be biased by the ready purification of actin from skeletal muscle and by the difficulty in purifying a particular isoform away from others. However, tissue or cells which contain only one isoform exist, and purification of actin from these sources eliminates the need to separate the highly similar isoforms from each other (Shuster & Herman, 1995; Strzelecka-Golaszewska et al., 1985). Ezrin (Shuster & Herman, 1995), profilin (Segura & Lindberg, 1984), thymosin β-4 (Weber et al., 1992), and l-plastin (Namba et al., 1992) have been shown to interact more strongly with β-actin than with rabbit skeletal muscle α-actin. The mechanism behind this isoform selectivity is unclear, though the differences in charged residues at the amino terminus of actin have been suggested as a possible mechanism. In contrast, von Arx et al. (1995) suggest that the differences in the N-terminal 83 amino acids of actin, which contain the sequences most divergent among isoforms, do not contribute to the observed suppression of contractile phenotype by expression of cytoplasmic actins in cardiac myocytes.

In order to begin to address the isoform specific functions of actins, we have examined several chemical and physical properties of filaments of purified actin isotypes. We find differences in the binding and unbinding of phalloidin to these actins, in the bulk rheologic properties of isoactin solutions, and in the lengths of isoactin filaments polymerized in the presence of phalloidin. These differences are discussed in light of the known sequence differences of the isoforms.

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MATERIALS AND METHODS

Salts, buffers, ATP, DTT, phalloidin, and tetramethylrhodamine isothiocyanate-labeled (TRITC) phalloidins were obtained from Sigma (St. Louis, MO).

Proteins. Rabbit skeletal muscle α-actin and bovine α-smooth muscle actins were purified from acetone powders as described in Chaponnier et al. (1995) (Strzelecka-Golaszewska et al., 1985). Chicken gizzard γ-smooth muscle actin was purified as described (Strzelecka-Golaszewska et al., 1980). Bovine erythrocyte β-actin and chicken skeletal muscle α-actins were purified as described in Schuster and Herman (1995). Native yeast actin and the actin mutant act1−135 (E4A) (Wertman et al., 1992) were purified by affinity chromatography against DNase (Lazarides & Lindberg, 1974), eluted with formamide, and separated from the formamide by gel filtration on G-25 Sephadex (Li et al., 1995). Native yeast actin was a gift from R. Li of Harvard Medical School.

To control for the potential effects of formamide exposure in generating differences in phalloidin binding kinetics, purified rabbit skeletal muscle actin at 1 mg/mL was exposed to 50% formamide in 2 mM Tris (pH 8.0), 0.5 mM ATP, 0.2 mM $CaCl_2$, and 0.2 mM DTT (G buffer) for 5 min, a time similar to that used during the preparation of yeast actin. Actin was separated from the formamide by gel filtration chromatography on a Pharmacia FPLC fast-desalting column equilibrated with G buffer. Gel-filtered actin was polymerized by the addition of salts to 150 mM KCl, 2 mM $MgCl_2$, 2 mM Tris (pH 8.0), 0.5 mM ATP, 0.2 mM $CaCl_2$, and 0.2 mM DTT (F buffer) and centrifuged at 100000*g*, and the pellet was resuspended and dialyzed overnight against G buffer. After 24 h of dialysis, the G-actin was clarified by centrifugation at 100000*g* and the actin concentration determined from the absorbance at 290 nm. Actin was subsequently polymerized and used at concentrations of 0.6−1 mg/mL.

Kinetic Analysis of Phalloidin Binding. Analysis of the binding of TRITC−phalloidin to various actin isoforms was carried out as described in Allen and Janmey (1994). The fluorescence enhancement of TRITC−phalloidin in the presence of F-actin was interpreted to be kinetically equivalent to the binding of the phalloidin molecule to actin (Allen & Janmey, 1994; De La Cruz & Pollard, 1994; Huang et al., 1992). The fluorescence enhancement was measured in a Perkin-Elmer LS-50b fluorescence spectrophotometer and the change in fluorescence fit to the equation

$$[^{\Delta}\text{TRITC}-\text{phalloidin}-\text{F-actin complex}] = B(1 - e^{-k_+[\text{TRITC}-\text{phalloidin}]\tau})$$

where B is a constant which relates the fluorescence value to the initial concentration of F-actin, $^{\Delta}$TRITC−phalloidin is the enhanced emission form of TRITC−phalloidin and τ is time, using the Kaleidagraph 3.0 software program. Dissociation of TRITC−phalloidin was determined upon addition of a 20−25-fold excess of unlabeled phalloidin to the TRITC−phalloidin-saturated actin filaments. We observed that lower concentrations of unlabeled phalloidin did not prevent rebinding of the TRITC−phalloidin and gave an underestimate of the dissociation rate (data not shown). The dissociation rate constant was calculated by fitting the loss of fluorescence to the equation

$$[^{\Delta}\text{TRITC}-\text{phalloidin}-\text{F-actin complex}] = Ce^{-k-\tau}$$

where C is a constant that relates the fluorescence value to the initial concentration of the $^{\Delta}$TRITC−phalloidin−F-actin complex.

Rheologic Measurements. The bulk viscoelastic properties of purified actin isoforms were measured using a Rheometrics RFSII fluid spectrometer. Samples (700 μL) of 0.6 g/L actin were measured using cone and plate geometry. Salts were added to G-actin solutions to bring the buffer conditions to 150 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, and 10 mM Tris (pH 7.4), and the sample was added to the rheometer within 15 s. It was important to add the solution to the rheometer in less than 1 min, as pipetting the solution at longer time periods reduced the ratio of G' to G''. The time dependent changes in G' and G'' after addition of salts to monomeric actin were measured at a frequency of 10 rad s^{-1} and a strain of 1%. The frequency and strain dependence of polymerized actins were measured 1 h after initiation of polymerization. All measurements were done at room temperature.

Visualization of Actin Filaments. Actin was polymerized in the presence of TRITC−phalloidin at concentrations of 5 μM each, diluted to a final concentration of 5 nM, and visualized on a Zeiss IM 405 inverted fluorescence microscope. Filaments were imaged with an Dage-MTI (Michigan City, IN) SIT camera and recorded to videotape on a S-VHS video recorder and images captured with a Pixelpipeline video frame grabber card (Perceptics, Nashville, TN) in a Macintosh IIci computer using NIH Image 1.49 (Wayne Rasband, National Institutes of Health, Bethesda, MD). Filament lengths were measured on 30 or more filaments by thresholding the image of the filament, finding the optical center of the filament (Käs et al., 1996), counting the number of pixels in the single pixel trace of the filament length, and multiplying by a scaling factor which converts the number of pixels to a length in microns. The scaling factor was determined by counting the number of pixels per 10 μm length on a calibrated slide.

Molecular Graphics and Sequence Analysis. Models of the actin filaments were generated using the MacImdad software package on a PowerMac 7100 (Apple Computer). Atomic coordinates for the monomers within the actin filament were obtained from Michael Lorenz (Lorenz et al., 1993). Protein sequences were taken from the WWW-Entrez protein data bank of The National Center for Biotechnology Information of the National Library of Medicine and the NIH.

RESULTS

Phalloidin Binding Kinetics. Phalloidin binding is a common characteristic of filamentous actin, and we have determined the binding and unbinding kinetics of TRITC−phalloidin to the various actin isoforms. As previously shown for rabbit skeletal muscle α-actin (Allen & Janmey, 1994; De La Cruz & Pollard, 1994), phalloidin binding to all isoactins was first-order for actin and for phalloidin. Phalloidin dissociation was also a first-order reaction. The dissociation of TRITC−phalloidin from F-actin, measured

[1] Abbreviations: TRITC−phalloidin, tetramethylrhodamine isothiocyanate-coupled phalloidin; DTT, dithiothreitol; F-actin, filamentous actin; G-actin, monomeric actin; G', storage modulus; G'', loss modulus.

Table 1: Association and Dissociation Rate Constants for the Binding of TRITC-Phalloidin to Various Isoactins[a]

actin type and source	k_+ ($\times 10^{-3}$ M^{-1} s^{-1}) (standard error; *N*)	k_- ($\times 10^{3}$ s^{-1}) (standard error; *N*)	K_d (nM)
rabbit α-skeletal muscle	29 (4; 12)	0.48 (0.01; 6)	16
formamide-treated	30 (5; 3)	0.60	20
chicken α-skeletal muscle	30 (8; 3)	0.48 (0.03; 3)	16
bovine α-smooth muscle	29 (5; 8)	0.49 (0.04; 4)	17
bovine *β*-erythrocyte	68 (5; 8)	0.48 (0.01; 3)	7
Saccharomyces cerevisiae	70 (3; 3)	3.5 (2; 4)	50
S. cerevisiae E4A mutant	71 (3; 3)	5.3 (2.2; 3)	75

[a] The rate of TRITC−phalloidin association and dissociation from purified isoactins was measured at differing concentrations of actin and TRITC−phalloidin, and the rate constants were calculated as described in Materials and Methods. Rabbit skeletal muscle actin was treated with 50% formamide, as described in Materials and Methods, to control for potential differences generated by the different preparative methods.

as described in Materials and Methods, is identical for actins derived from higher eukaryotes (bovine, rabbit, and chicken) (Table 1). In contrast, TRITC−phalloidin dissociates from yeast actin several times faster than from the mammalian actins tested. As indicated by the increased standard error, there was more experimental variation in the measured phalloidin dissociation kinetics of yeast actin compared with that of mammalian actins. The faster off rate from yeast actins is not due to exposure to formamide during purification, as rabbit skeletal muscle actin treated similarly did not have altered phalloidin binding constants.

TRITC−phalloidin association kinetics differ among actin isoforms. α-Actins from skeletal and smooth muscle sources bind phalloidin at approximately $^1/_2$ of the rate of cytoplasmic *β*-actin or yeast actins. The differences in the number of charged residues at the N terminus of actin do not appear to correlate with phalloidin binding kinetics, as the mutation of glutamic acid to alanine at position 4 of yeast actin (Act-135; Wertman et al., 1992) did not alter the phalloidin binding constants.

Rheologic Properties. Since one physiologic role of actin is providing mechanical strength to the cytoplasm, specific functions of actin isoforms may relate to differences in viscoelasticity, as measured by rheology. Rheologic properties are characterized by both elastic and viscous contributions, and a common criterion for gel formation is that the elastic modulus exceed the viscous or loss modulus over a range of times and frequencies (Almdal et al., 1993). We characterized the viscoelastic characteristics of 0.6−1.0 mg/mL solutions of five actin isoforms. The time dependent changes in the storage and loss moduli shear (G' and G'', respectively) were measured after addition of salts to a final concentration of 150 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.2 mM DTT, and 10 mM TRIS (pH 7.4) (Figure 1). Muscle actins purified from either rabbit or chicken formed gels ($G' > G''$) as they polymerized, with skeletal muscle actins having the highest values of G' and the highest ratio of G' to G''. Smooth muscle α- and *γ*-actins also formed gels but with significantly lower values of G' and G''. In contrast, bovine erythrocyte *β*-actin did not form a gel under these conditions, with G' and G'' being equivalent. Addition of phalloidin to *β*-actin did not increase the ratio of G' to G'' (data not shown).

Previous reports have demonstrated that muscle α-actin gels increase in elastic resistance at increasing but still modest deformations before losing their elasticity at strains of >20% (Janmey et al., 1988; MacKintosh et al., 1995). Both rabbit and chicken skeletal muscle α-actin gels showed significant strain hardening between 5 and 15−20% strain followed by a rapid loss of G'. The reduction in G' was not reversible if the sample was subsequently measured at low strain (data not shown). In contrast, smooth muscle α- and *γ*-actins lacked strain hardening and show a gradual loss of G' with increasing strain. G'' did not change significantly with increasing strain in muscle actin samples. *β*-Actin showed a gradual loss of G' and G'', with G'' becoming greater than G' at strains larger than 5−10%.

The frequency dependent viscoelastic properties of the various actin isoforms were also analyzed. Below 10 rad/s, G' and G'' were nearly frequency independent (data not shown) At frequencies higher that 10 rad/s, G'' increased significantly in skeletal muscle α-actin but less so in other muscle isoforms. G' and G'' of cytoplasmic *β*-actin solutions increased several-fold as the frequency increased, but the elastic modulus did not reach the level of α- or *γ*-actins.

Single Isoactin Filaments in Solution. The large differences in the viscoelastic properties of isoactin solutions lead us to examine the characteristics of individual filaments free in solution. All samples of isoactins polymerized in the presence of TRITC−phalloidin had filaments of lengths 1 *μ*m and longer detectable by fluorescence microscopy (Figure 2). In accordance with previously published work (Burlacu et al., 1992; Kaufmann et al., 1992), large numbers of filaments of skeletal muscle actin from rabbit and chicken, polymerized in the presence of TRITC−phalloidin, had lengths of 5 *μ*m or greater (Table 2). In contrast, smooth muscle α-actin and gizzard *γ*-actin formed filaments which had an average length of 2−3 *μ*m. Erythrocyte *β*-actin polymerized in the presence of TRITC−phalloidin also formed filaments with an average length similar to that of chicken skeletal muscle.

Preliminary examination of thermal fluctuations of TRITC−phalloidin-labeled α- and *γ*-actin filaments (Figure 2A′−C′) showed smooth undulations similar to those previously reported for rabbit skeletal muscle actin (Käs et al., 1996). In contrast, *β*-actin (Figure 2D′) showed small regions of high curvature surrounded by large straight domains. These hingelike motions have not been previously reported to occur in actin filaments, and continuing work aims to address the nature of these movements.

DISCUSSION

Different isoforms of actin exist within a single cell, and evidence is mounting that each isoform may have different but overlapping functions within the cell. Actin isotypes are often segregated to different regions of the cytoplasm, with sorting probably occurring through both the localization of mRNA expression and the localization of the expressed

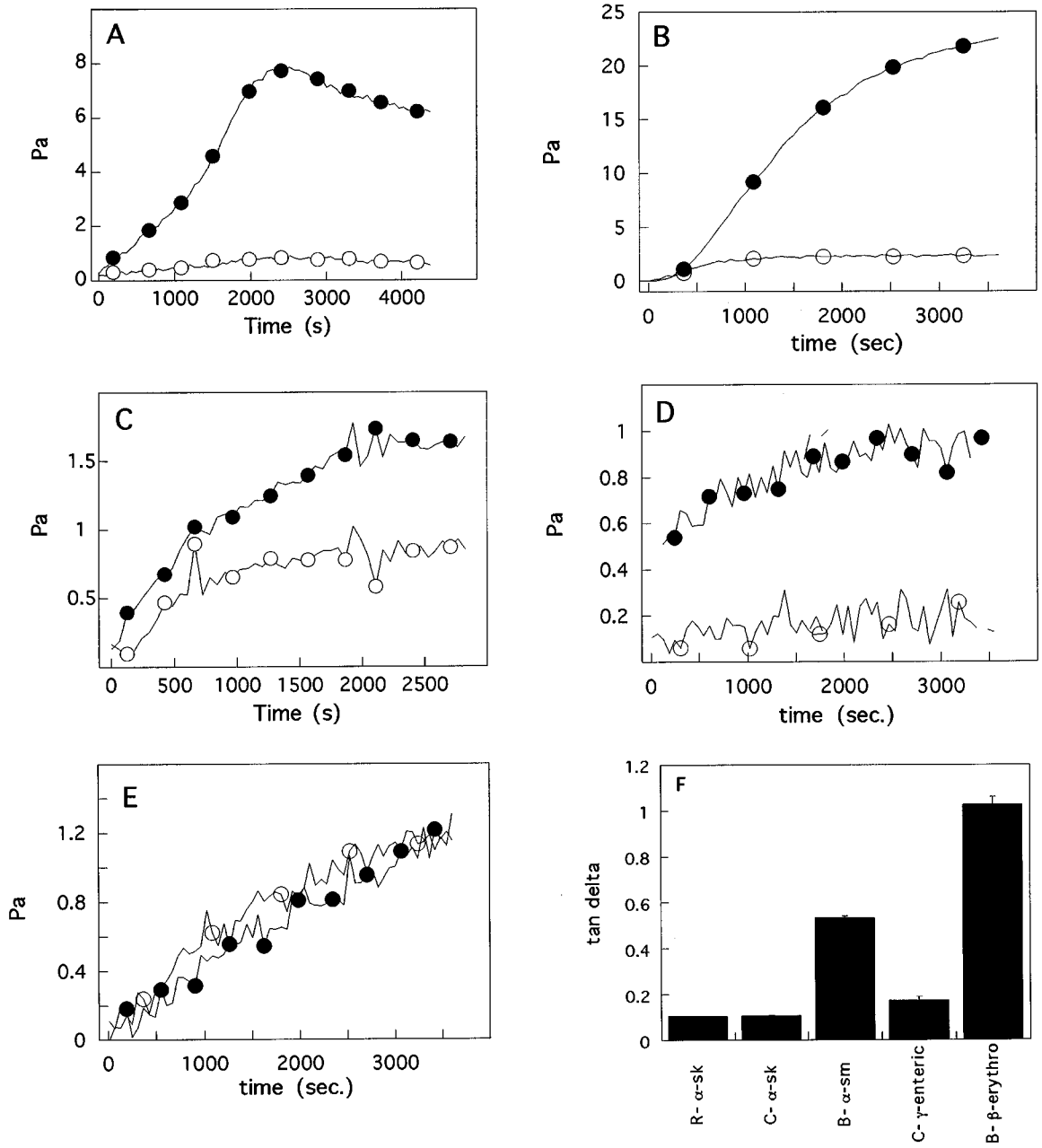

FIGURE 1: Viscoelastic properties of polymerized isoactins. Purified isoactins were polymerized by the addition of 150 mM KCl and 2 mM $MgCl_2$ (final concentration) on the rheometer plates and the changes in storage (G', closed circles) and loss (G'', open circles) moduli in pascal (Pa, or N/m^2) recorded continuously over time as described in Materials and Methods, with symbols added to identify the data. The isoactins were (A) 0.7 g/L rabbit α-skeletal muscle actin, (B) 0.6 g/L chicken α-skeletal muscle actin, (C) 1 g/L bovine α-smooth muscle actin, (D) 0.6 g/L chicken gizzard γ-smooth muscle actin, and (E) 0.6 g/L bovine erythrocyte β-actin. (F) The loss tangent of each isoactin at the end of the experiment is illustrated. Each value is the average of eight measurements of G' and G'' and the error bars indicate the standard error of the mean.

protein [see Herman (1993) for review]. Furthermore, different isoforms can be found in different actin rich structures, with α-actins more likely to be observed in stress fibers and β-actin enriched in the cell cortex (DeNofrio et al., 1989). The differential segregation of actin isoforms is likely due to interactions with actin binding proteins, some of which have a stronger affinity for one isotype over another.

As an initial approach to addressing differences in isoactin structure and function, we have measured two characteristic properties, phalloidin binding and bulk viscoelasticity, of filaments of purified actin isoforms. The differences in these properties suggest that there are significant but subtle structural differences between isoactins that may be critical to their cellular function.

Phalloidin Binding Kinetics. The specific binding of the mushroom toxin phalloidin is a common characteristic of filamentous actin. Though a mutant actin exists which does not bind phalloidin (Drubin et al., 1993), binding of derivatized phalloidins is used to localize actin filaments in cells ranging from plants to fungi to mammals [see Sheterline et al. (1995) for review]. We and others have measured the association and dissociation of rhodamine or TRITC−phalloidin to rabbit skeletal muscle actin and found that both association and dissociation are very slow processes with a k_+ of 3×10^4 M^{-1} s^{-1}, and a k_- of 5×10^{-4} s^{-1}(Allen & Janmey, 1994; De La Cruz & Pollard, 1994). This binding rate is many orders of magnitude slower than the potential collision rate, which led De La Cruz and Pollard (1994) to

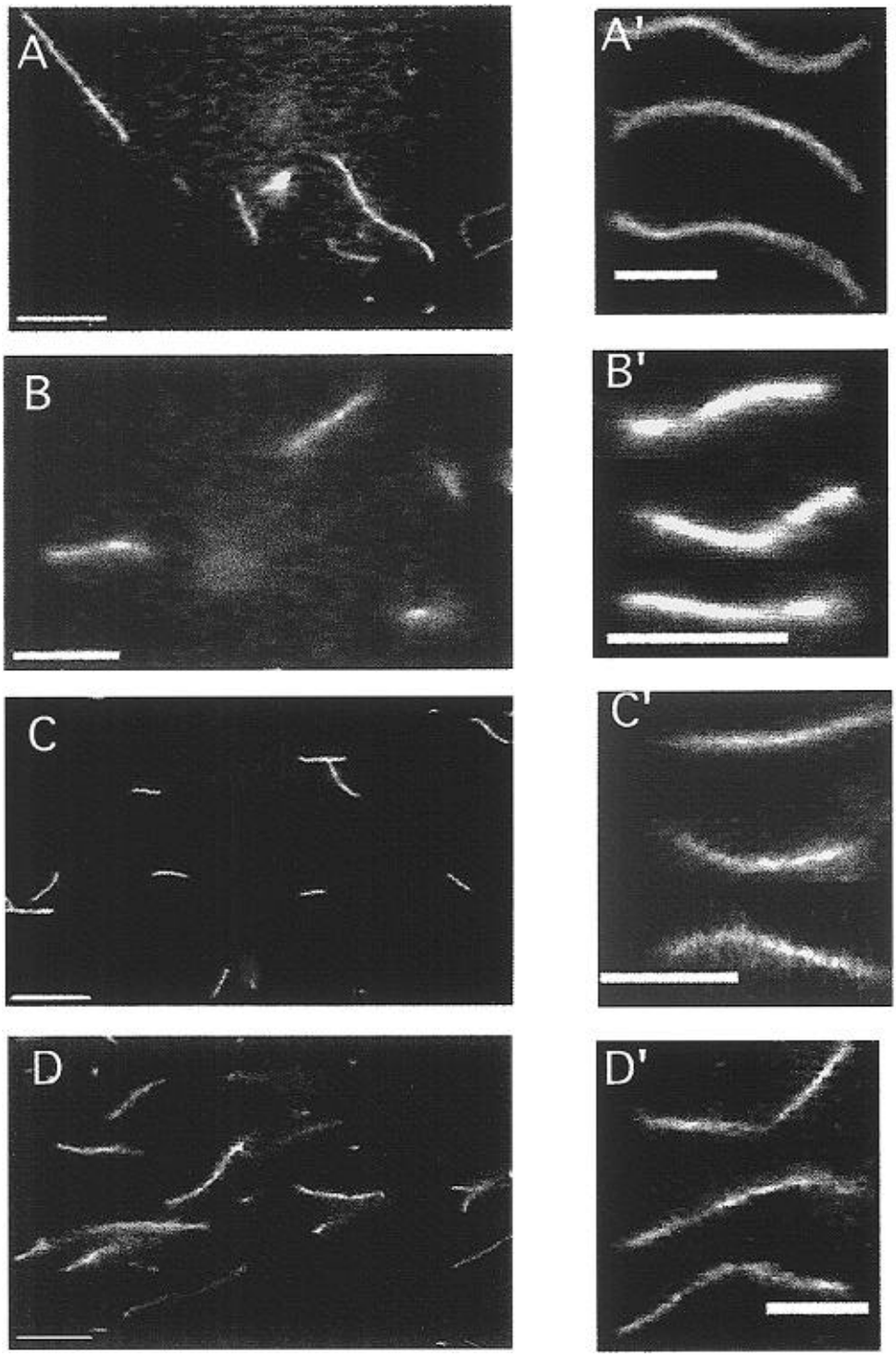

FIGURE 2: Visualization of isoactin filaments. Individual fields of purified (A) chicken α-skeletal actin, (B) bovine α-smooth muscle actin, (C) chicken gizzard γ- and (D) bovine erythrocyte β-actin were polymerized in the presence of TRITC-phalloidin and visualized as described in Materials and Methods to assay potential differences in filament lengths. Panels A′−D′ demonstrate the change in contour due to thermal fluctuations of single isoactin filaments at 0.2 s intervals. The bar in each figure is 5 μm.

hypothesize that the actin filament must breathe for phalloidin to access its binding site. Phalloidin is exposed to the solute in the deduced structure of the phalloidin-stabilized actin filament (Lorenz et al., 1993), but it is not clear how accessible the binding site is in native filaments. Furthermore, the possibility that three independent monomers contribute to the phalloidin binding site may minimize the frequency at which the protein chains are in the correct orientation for phalloidin binding. If the filament breathing hypothesis is correct, the phalloidin binding kinetics reflect underlying structural dynamics of the filament and differences in the phalloidin binding kinetics reflect differences in the dynamics of monomer−monomer interactions within the filament.

We report a 2-fold higher affinity of β-actin for phalloidin when compared to measurements from this (Allen & Janmey, 1994) and other labs (De La Cruz & Pollard, 1994) on skeletal muscle α-actin. The increased affinity is due completely to a 2-fold increase in the association rate constant for phalloidin binding (Table 1). A similar 2-fold increase in the association rate constant was measured for the other nonmuscle actin we analyzed, yeast actin. In contrast, De

Table 2: Average Length, Storage Modulus, and Loss Tangent of Isoactin Filament Solutions[a]

actin type	average length (μm) (standard deviation, $N > 30$)	G' (Pa)	loss tangent
rabbit α-skeletal muscle	n.d.	7	0.10
chicken α-skeletal muscle	4.6 (1.7)	22	0.11
bovine α-smooth muscle	2.7 (0.8)	1.5	0.52
chicken γ-gizzard	1.6 (0.4)	0.9	0.17
bovine β-erythrocyte	5.2 (2.1)	1.0	1.0

[a] Actin filament lengths of isoactins polymerized in the presence of TRITC−phalloidin were determined as described in Materials and Methods. G' and the loss tangent were taken from the data in Figure 1 at 2500 s after initiation of polymerization. [b] nd means no data from this study.

La Cruz and Pollard (1996) report an association rate constant for phalloidin binding to *Acanthamoeba* actin that is similar to that of rabbit skeletal muscle α-actin. The differences in association rate constants do not correlate simply with the charge differences at the N terminus of various actins, as both native yeast actin and an actin mutant, glutamic acid at position 4 substituted by alanine (Drubin et al., 1993), have identical association rate constants. However, this does not exclude the possibility that phalloidin binding rates are influenced in part by the sequence differences at the N terminus, as small changes in the actin sequence can have long range effects on actin structure.

Rheologic Properties. The viscoelastic properties of a non-cross-linked polymer solution are defined by a complicated relationship among concentration, flexibility, and length. We observed a range of rheologic properties from the actin isoforms tested. In accordance with previous measurements from this laboratory as well as others (Janmey et al., 1994), α-actin prepared from rabbit or chicken skeletal muscle polymerized to form solutions with significant elasticity at concentrations of 0.6−1 mg/mL, and with equally low loss tangents (ratio of G'' to G'). These samples showed strain hardening at strains greater than 5% and broke irreversibly at strains beyond 15−20%. α- and γ-smooth muscle actins also formed viscoelastic gels, but with lower shear moduli and greater loss tangents than the skeletal muscle isoforms. β-Actin at this relatively low concentration polymerized to form highly viscous solutions but did not develop an elastic modulus (G') greater than its loss modulus (G''), even in the presence of phalloidin to stabilize filaments.

The bulk elasticity of an actin filament solution depends very strongly on filament length (Janmey et al., 1988; MacKintosh et al., 1995), and the reduction in average filament length observed in α-smooth muscle and γ-actins polymerized in the presence of phalloidin can explain the decreased elasticity observed. In the absence of phalloidin, average filament lengths are expected to be less than or equal to that polymerized with phalloidin. The viscoelasticity of β-actin solutions is harder to interpret simply on the basis of polymer length. β-Actin solutions at the low concentration utilized did not form a gel when polymerized with or without phalloidin. Yet, in the presence of phalloidin, β-actin polymerizes to form filaments of the same approximate lengths as those of α-skeletal muscle actin from chicken (Table 2), which was highly elastic. This finding and our observation of unusual movements in β-actin filaments (Figure 2) suggest that other parameters which contribute to the elastic properties of actin solutions, such as filament stiffness (MacKintosh et al., 1995), may differ between α- and β-actins.

The differences in viscoelasticity are likely to reflect differences in the solution structures of the filaments but are not necessarily representative of rheologic differences in vivo. Practical limits on protein purification, and the onset of nematic phase transitions for F-actin at high concentrations in vitro (Suzuki et al., 1991), limit the current studies to actin filaments of long but unregulated length and to concentrations nearly 1 order of magnitude lower than reported in cells. Therefore, while the large, reproducible difference in elasticity between α-, γ-, and β-actins strongly suggests that these actins differ in either filament stability, as reflected in length (Erickson, 1989), or flexibility, the present data are insufficient to conclude that networks of β-actin may not be highly elastic at higher concentrations. Actin filaments in cells also differ from purified F-actin in vitro by being modulated by actin binding proteins that alter filament association through cross-linking and bundling and filament length through capping, severing and actin monomer sequestration [see Sheterline et al. (1995) for review]. These actin binding proteins, particularly actin filament cross-linking proteins, can induce short actin filaments to form elastic gels that resemble gels formed in vitro through the entanglements of long filaments (Janmey et al., 1990). In the presence of cross-linkers at concentrations found in cells, filament length ceases to contribute to actin's elastic properties, and properties such as filament stiffness and cross-linker dynamics become much more important (MacKintosh et al., 1995).

The differences in the behavior of β-actin filaments we observe may be relevant to its cellular functions. β-Actin is localized to regions of protrusive activity, and models of protrusion that use rectified thermal noise as the engine of membrane protrusion (thermal ratchets) (Peskin et al., 1993) are sensitive to the stiffness of the rectifying filaments. At some level, these isoactin differences in breathing or flexibility must influence the interactions of actin binding proteins with the actin filament, contributing to the preference of various actin binding proteins for β- instead of α-actins. In the cell, this could allow differential regulation of actin dynamics in compartments rich in a particular isoactin.

Sequence Variation and Phalloidin Binding. The phalloidin association and dissociation rate constants were observed to vary among the isoactins we analyzed. We observed that several of the amino acids that differ between the cytoplasmic and muscle actins are at or near the phalloidin binding sites, as illustrated in Figure 3. The semiconservative changes between α-muscle and cytoplasmic actins illustrated in Figure 3 are I-76 to V, T-103 to V, M-176 to L, V-201 to T or S, N-244 to Q, T-260 to A, I-288 to V, and A-365 to S. Of these, I-76, M-176, and V-201 are all very close to the binding sites for phalloidin (see Figure 3). Consistent with the change at M-176 being important, mutations of amino acids R-177 and D-179 to A in yeast lead to a loss of phalloidin binding (Drubin et al., 1993). V-201 (T in cytoplasmic β- and S in yeast actin) is at the

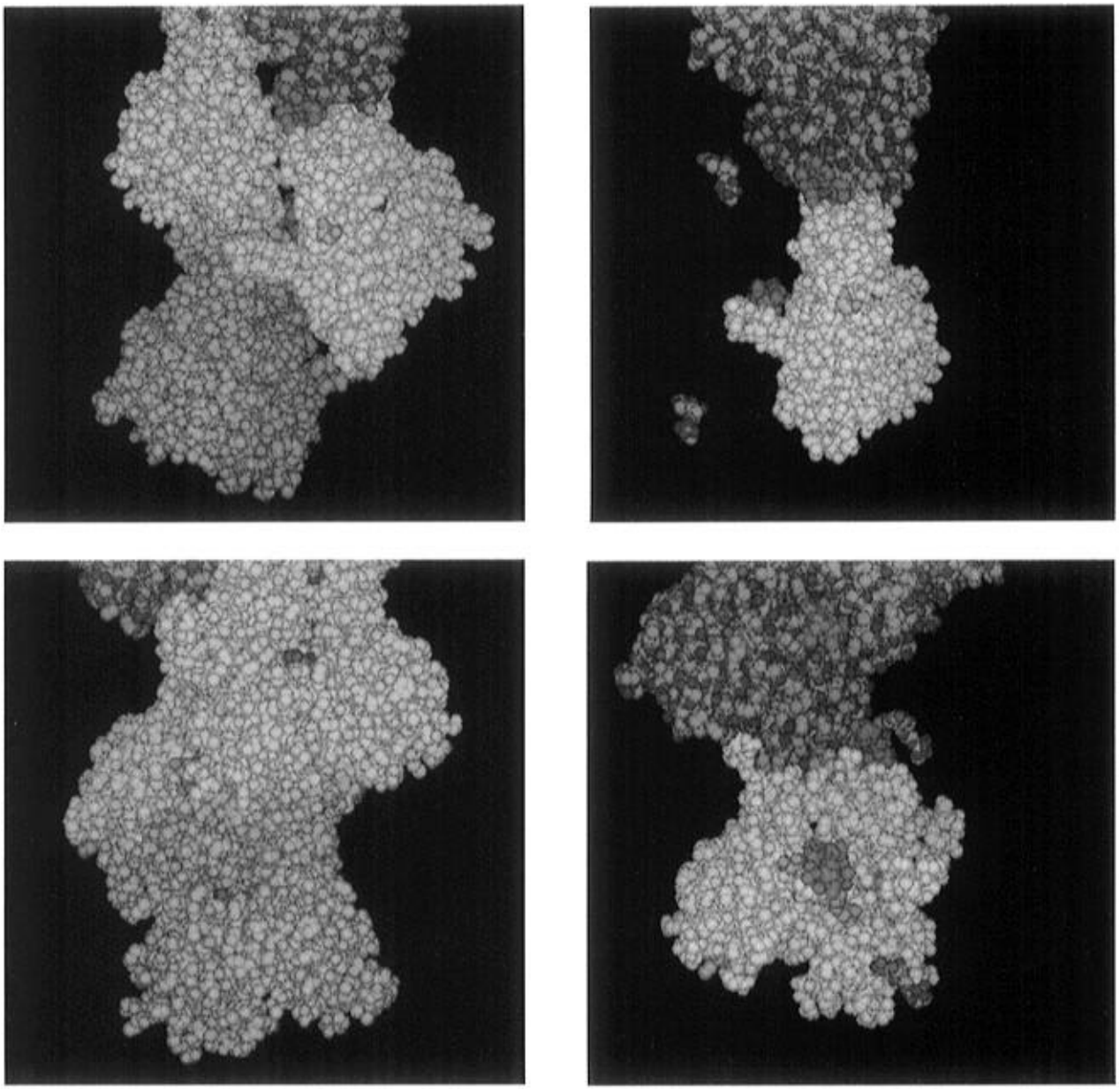

FIGURE 3: Localization of conserved differences between cytoplasmic and muscle actins. Consistent amino acid differences between muscle and nonmuscle actins are illustrated in the molecular model of the actin filament. The left-hand panels show two orientations of the monomers within the filament. Each monomer is colored differently (yellow, light gray, light blue, and red-blue-gray) for clarity. The N terminus of the barbed-end terminal actin (yellow) is colored red as a position marker. Consistent variations in sequence between muscle and nonmuscle actins are highlighted in purple (monomer 1) and green (monomer 2). In the right-hand panels, the monomers from one of the long-pitch helices have been removed to better visualize the interior domains of the filament. A phalloidin molecule can now be observed near the hydrophobic loop. The nucleotide molecules from the invisible monomers have been included to provide orientation information.

interface of two monomers within the long-pitch helix of the filament. The substitution of T or S might allow more rapid breathing and phalloidin binding. However, this position is also T in *Acanthamoeba*, which is reported to have an association rate constant equal to that of rabbit α-skeletal muscle actin (De La Cruz & Pollard, 1996). This suggests that interactions at a single position in the filament are unlikely to be the sole determinants of filament breathing and the rate of phalloidin association. The position of this residue at the junction of two monomers within the long-pitch helix does, however, lend credence to the hypothesis that monomer−monomer breathing within the filament defines the rate of phalloidin binding (De La Cruz & Pollard, 1996).

The dissociation of phalloidin from yeast actin is approximately 1 order of magnitude more rapid than that from mammalian actins. From comparative sequence analysis, it is not possible to conclude exactly which of the amino acid differences leads to this rapid dissociation. Yeast and mammalian actins are identical in sequence in the 10 amino acids to either side of V-76 and have only one change, T-194 to S, in the 10 amino acids to either side of position 201. There are four changes around position 176: E-167 to A, Y-169 to F, A-170 to S, and L-178 to I. While these differences are suggestive, there are also differences in sequence between mammalian and yeast actins in the "hydrophobic plug", amino acids 256−274 (Holmes et al., 1990). This structure is thought to form a bridge between the two long-pitch helices, and mutations in this region destabilize the filament in vitro (Chen et al., 1993). In the context of the suggestion that intramonomer movements are important in providing access to the phalloidin binding site,

such movements may also be important in allowing phalloidin dissociation.

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