Binding of Dystrophin's Tandem Calponin Homology Domain to F-Actin Is Modulated by Actin's Structure

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ABSTRACT Dystrophin has been shown to be associated in cells with actin bundles. Dys-246, an N-terminal recombinant protein encoding the first 246 residues of dystrophin, includes two calponin-homology (CH) domains, and is similar to a large class of F-actin cross-linking proteins including α-actinin, fimbrin, and spectrin. It has been shown that expression or microinjection of amino-terminal fragments of dystrophin or the closely related utrophin resulted in the localization of these protein domains to actin bundles. However, in vitro studies have failed to detect any bundling of actin by either intact dystrophin or Dys-246. We show here that the structure of F-actin can be modulated so that there are two modes of Dys-246 binding, from bundling actin filaments to only binding to single filaments. The changes in F-actin structure that allow Dys-246 to bundle filaments are induced by covalent modification of Cys-374, proteolytic cleavage of F-actin's C-terminus, mutation of yeast actin's N-terminus, and different buffers. The present results suggest that F-actin's structural state can have a large influence on the nature of actin's interaction with other proteins, and these different states need to be considered when conducting in vitro assays.

INTRODUCTION

Dystrophin is the 3685-residue protein product of the gene defective in Duchenne muscular dystrophy, and it is found in the muscle membrane cytoskeleton (Bonilla et al., 1988). Dystrophin shares significant homology with actin-binding proteins such as α-actinin and spectrin, and the amino-terminus of dystrophin has been shown to be functionally homologous to the amino-terminus of α-actinin (Hemmings et al., 1992). Dystrophin has been shown to be associated in cells with actin bundles, and is thought to form a structural linkage between the actin-based cytoskeleton and the extracellular matrix (Tidball and Law, 1991; Belkin and Burridge, 1995). The two CH domains within the N-terminal region of dystrophin may form a single actin-binding domain, based upon analysis of related proteins (Puius et al., 1998; Stradal et al., 1998). It has been shown using electron microscopy (Tidball and Law, 1991) and immunofluorescence (Belkin and Burridge, 1995) that dystrophin is associated with actin bundles located near the sarcolemmal membrane, and it has been suggested that dystrophin may form antiparallel dimers that cross-link different actin filaments (Ahn and Kunkel, 1993).

Dys-246, an N-terminal recombinant protein encoding the first 246 residues of dystrophin (Rybakova et al., 1996; Renley et al., 1998), includes two calponin-homology (CH) domains, and is similar to a large class of F-actin cross-linking proteins including α-actinin, fimbrin, and spectrin (Matsudaira, 1991; Puius et al., 1998). It has been shown that this fragment binds to F-actin in vitro (Rybakova et al., 1996; Renley et al., 1998). Expression or microinjection of amino-terminal fragments of dystrophin (Hemmings et al., 1992) or the closely related utrophin (Winder et al., 1995) resulted in the localization of these protein domains to actin bundles. However, neither intact dystrophin nor N-terminal recombinant fragments have been observed to bundle actin filaments (Rybakova et al., 1996; Rybakova and Ervasti, unpublished results) in vitro. By analogy with actin cross-linking proteins such as α-actinin, it would be expected that actin bundling would be due to the formation of a dystrophin dimer. The possible dimerization of either intact dystrophin or N-terminal fragments of dystrophin has been controversial. Dimers of dystrophin were observed by electron microscopy (Pons et al., 1990), but not observed by immunoprecipitation and blot overlays (Chan and Kunkel, 1997) or gel filtration and analytical ultracentrifugation (Rybakova and Ervasti, 1997). A recent study has found dimers of the N-terminal actin-binding domain both in crystals and in solution (Norwood et al., 2000).

METHODS

Actin prepared from rabbit skeletal muscle was purified on either a Sephadex G-200 column or a Superdex-200 column using the AKTA-Explorer system (Pharmacia, Uppsala, Sweden). G-Ca^{2+}-actin at 0.5 mg/ml was polymerized by 0.1 M KCl, and F-actin was diluted to 3 μM by 100 mM KCl, 10 mM Tris-HCl, Hepes, Mops, or Pipes buffer, pH 7.7, at room temperature. Trypsin-cleaved actin and erythrosin-actin were prepared as described (Orlova and Egelman, 1995; Orlova et al., 1995). Yeast actin was purified from wild-type and 4Ac-mutant yeast cells using a DNase I column method, as described (Prochniewicz and Thomas, 1999). The 4Ac

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mutant was a gift from Dr. Peter Rubenstein, and in this mutant three amino-terminal residues have been replaced by four residues from skeletal muscle actin (Cook et al., 1993).

Dys-246 was expressed and purified as previously described (Rybakova et al., 1996), but was step-dialyzed in buffer A (10 mM Tris-HCl, pH 8.0) containing 500 mM NaCl and 0.1 mM dithiothreitol, followed by buffer A containing 0.1 M NaCl and 1 mM iodoacetamide, and finally into buffer A containing 0.1 M NaCl. After concentration in a Centriplus, the Dys-246 was centrifuged immediately before use for 30 min at 100,000 $\times$ *g* to remove aggregated material.

Different actin samples (3 μM) in either Tris or Hepes buffer were incubated with Dys-246 (3–12 μM) at room temperature for 10–20 min. Sample mixtures were applied to a grid and stained with 1% uranyl acetate, and then examined in a JEOL 1200 EXII electron microscope at an accelerating voltage of 80 keV and a nominal magnification of 30,000×. Samples of the actin/Dys-246 complexes were examined directly, without any centrifugation of the mixtures.

Dys-246 binding to actin was measured using high- and low-speed sedimentation assays (Rybakova et al., 1996). Various amounts of Dys-246 were incubated at room temperature for 30 min with 3–6 μM F-actin in 10 mM Tris or Hepes buffer, 0.1 M KCl, pH 7.6, and centrifuged for 20 min at either 20,000 or 100,000 $\times$ *g*. The supernatants and resuspended pellets were electrophoresed in SDS on polyacrylamide gels, and these were stained with Coomassie blue and analyzed densitometrically using a Bio-Rad GS-670 imaging densitometer. Intensities of the scanned bands were quantitated by volume integration after background subtraction.

RESULTS

We have found that the mode of dystrophin's binding to F-actin can be switched between binding to isolated actin filaments and bundling of many filaments. Fig. 1 *a* shows an electron micrograph of skeletal muscle actin filaments incubated with Dys-246 in Tris buffer, where only the random binding of Dys-246 to single filaments can be observed. It has previously been shown that the dissociation constant for Dys-246 binding to actin in Tris buffer is ~14 μM (Renley et al., 1998). Under the conditions used (3 μM actin, 6 μM Dys-246) we would expect with this dissociation constant that only ~13% of the Dys-246 molecules would be bound to actin, and that most actin subunits would not have a Dys-246 bound. Thus, the large background of unbound Dys-246 molecules prevents one from easily observing the small number of bound ones.

Labeling of actin by the covalent attachment of erythrosin to Cys-374 has been shown to induce a change in F-actin structure (Orlova et al., 1995). When erythrosin-labeled F-actin is incubated with Dys-246 in Tris buffer, under conditions where no bundling is observed for unmodified F-actin (Fig. 1 *a*), bundling of the actin filaments occurs (Fig. 1 *b*).

The cleavage of two C-terminal residues from F-actin by trypsin (Fig. 1 *c*) also induces a conformational change in the filament (O'Donoghue et al., 1992; Orlova et al., 1995). We find (Fig. 1 *d*) that Dys-246 bundles trypsin-cleaved actin in Tris buffer, while no bundling occurs for wild-type filaments under the same conditions (Fig. 1 *a*), and no regular bundling occurs for the trypsin-cleaved actin filaments alone (Fig. 1 *c*).

When these same experiments with unmodified skeletal muscle actin are done in Hepes buffer, bundling of F-actin is seen (Fig. 1 *f*). We can exclude possible pH effects in the change from Tris to Hepes due to temperature or concentration changes, since all experiments (and buffer preparations) were done at the same temperature, and the buffer concentration was held relatively constant. The effect would be consistent with a change in either F-actin or Dys-246 due to the change in buffer. However, the other experiments show that the change must be occurring in the F-actin.

Consistent with this, we find that no bundling of wild-type yeast F-actin occurs with Dys-246 in Tris buffer (Fig. 1 *g*), but extensive bundling occurs with wild-type yeast actin in Hepes, Mops, or Pipes buffers (data not shown). A preliminary study shows extensive bundling in Tris buffer with a yeast mutant actin (4Ac) (Fig. 1 *h*), suggesting that it is a change in actin's structure that is responsible for the switch between these two modes of interaction.

The regular lateral spacing of the actin filaments induced by Dys-246 can be seen in Fig. 2, arguing against a non-specific effect of Dys-246 on the actin, or an actin-actin interaction such as that which occurs at high concentrations of divalent cations. When only two actin filaments are linked (Fig. 2 *a*), the cross-bridges are seen in the plane of the two filaments, spaced axially by roughly the distance of the actin crossovers, and the filaments are spaced laterally by ~160 Å. When more actin filaments are cross-linked into three-dimensional bundles (Fig. 2, *b*, *c*, and *d*), more closely spaced cross-bridges are seen in projection. This follows from the general rules for cross-bridging actin filaments (DeRosier and Tilney, 1982). Due to the fact that these bundles are not highly ordered, regular transverse stripes are not seen in larger bundles (Fig. 2 *e*) that may contain >10 actin filaments.

Could the observed bundling be an artifact of specimen preparation for electron microscopy? We have used a low-speed cosedimentation assay to quantitate the amount of actin bound with the fragment in bundles under different conditions. We found (Fig. 3) that no significant fraction (~2.9%) of the skeletal muscle actin in Tris buffer was in the pellet after low-speed centrifugation (20,000 $\times$ *g* for 20 min), as has been shown previously (Rybakova et al., 1996). But this fraction increased to 29% when Hepes buffer was used and 60% when erythrosin-modified actin was used in Tris buffer (Fig. 3). Previous control experiments showed that Dys-246 bound to F-actin in Tris buffer (measured at high *g*) with a stoichiometry of 1:1 and an apparent K_d of 14 μM (Renley et al., 1998). Given a total actin concentration of 6 μM, and a total Dys-246 concentration of 6 μM (the concentrations used for the experiments shown in Fig. 3), the 14 μM dissociation constant predicts that the concentration of free actin (not bound by Dys-246) will be 4.5 μM, or that only 25% of the actin is bound by Dys-246. Although quantitatively predicting the amount of actin filaments that would be bound in bundles is difficult or impossible, the

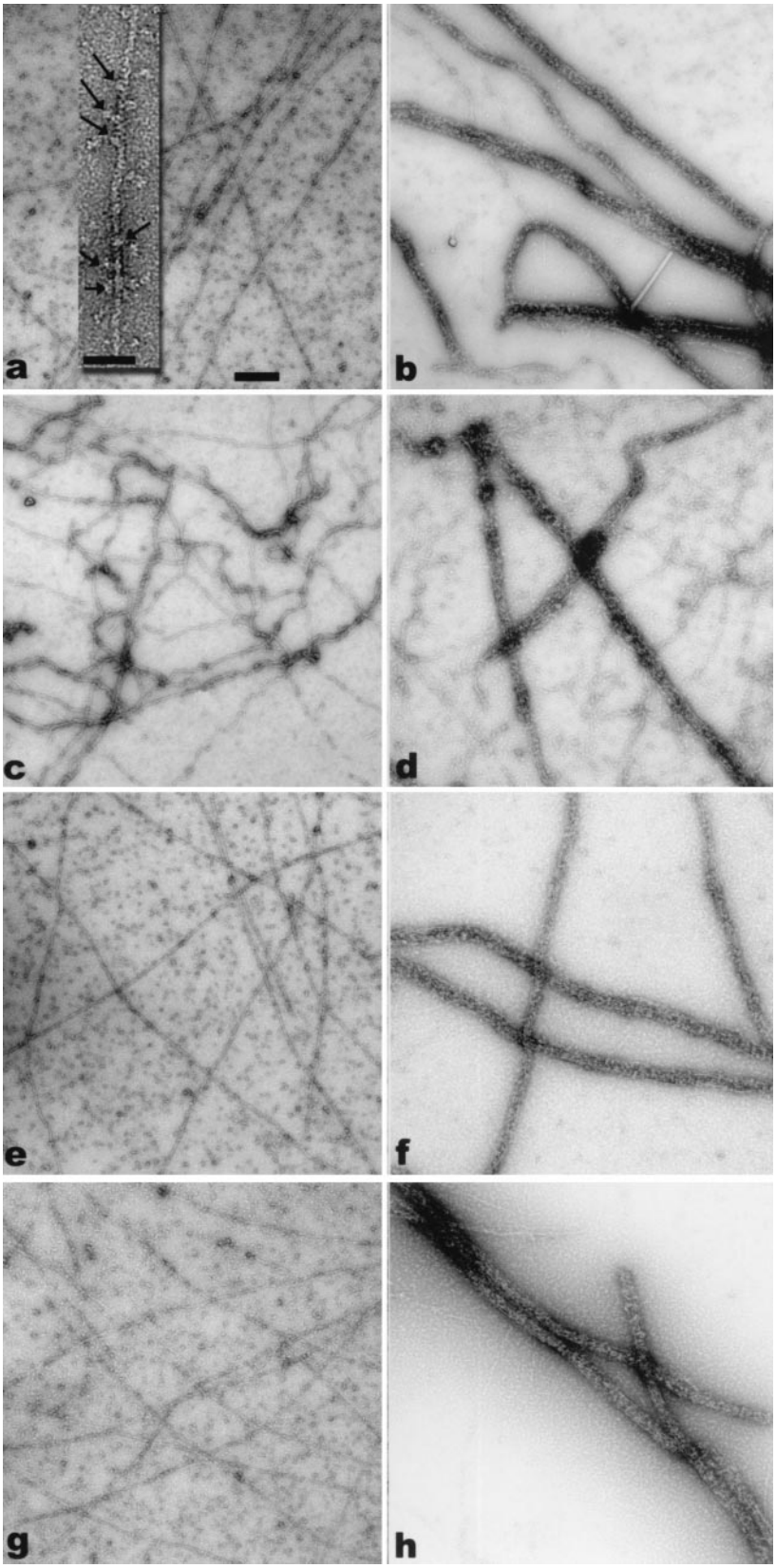

FIGURE 1 F-actin's structure can be switched from a state where Dys-246 binds only to single filaments (*a*, *e*, *g*), to a state where it bundles actin filaments (*b*, *d*, *f*, *h*). Skeletal muscle actin (3 μM) was incubated with Dys-246 (6 μM) in 0.1 M KCl and 10 mM Tris-HCl, pH 7.7. There is a random binding of Dys-246 to single actin filaments in Tris buffer (*a*), which can be seen more easily in the higher-magnification inset. When the actin filaments are covalently modified by the attachment of erythrosin at Cys-374 (Orlova et al., 1995, they are bundled by Dys-246 under the same conditions (*b*). Tryptically cleaved actin filaments are more fragile than normal filaments and tend to form small aggregates (*c*), but are bundled by Dys-246 in Tris buffer (*d*), under the same conditions where wild-type filaments are not bundled (*a*, *e*). When Tris buffer (*e*) is replaced by Hepes, skeletal muscle actin filaments are now bundled (*f*). Wild-type yeast actin (3 μM) was incubated with Dys-246 (7 μM) in 10 mM Tris buffer, and random binding is found to single filaments (*g*). Replacement of the first four amino acids with residues from skeletal muscle actin leads to a change in the mode of binding (*h*), where Dys-246 now cross-links the actin filaments into bundles. The scale bar in (*a*) is 1000 Å, and 500 Å within the inset.

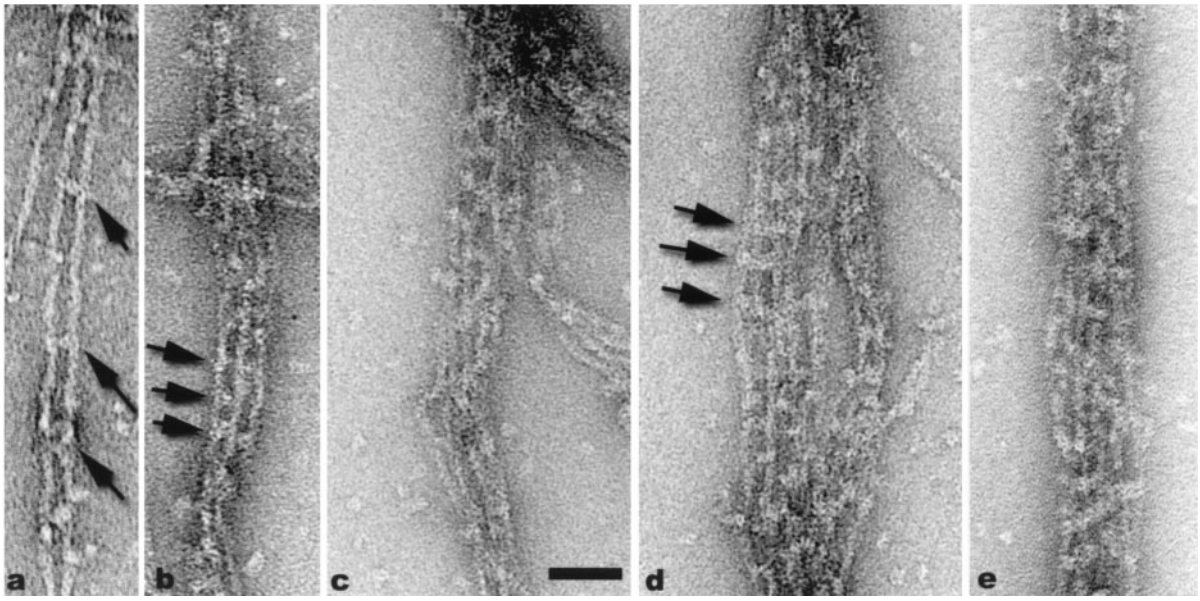

FIGURE 2 The specific nature of the Dys-246 cross-links can be seen. Skeletal muscle actin has been labeled with erythrosin and incubated with Dys-246. Individual cross-links between two filaments are clearly visible in (*a*), forming a ladder in the plane of the image. In *b*, *c*, and *d* the transverse bands produced by the Dys-246 cross-bridges can be seen in small three-dimensional bundles. Due to the disordered nature of these bundles, regular transverse bands are not seen in larger, three-dimensional bundles (*e*). The scale bar in (*c*) is 500 Å.

amount of actin seen in the low-speed spin pellet (bundled by Dys-246) is consistent with the measured affinity of Dys-246 for actin. The increased amount of actin seen in the erythrosin-actin pellet might be due to either an increased affinity of erythrosin-actin for Dys-246 or a greater cooperativity in bundle formation.

More importantly, could the bundling, seen in Hepes buffer, with erythrosin-actin, with tryptic-cleaved actin, and with the yeast 4Ac mutant, be due to an increase in the affinity of Dys-246 for actin? That is, could these changes in actin be associated with a greatly increased affinity of Dys-246 for actin, such that no bundling is seen when the binding is weak, but extensive bundling is seen when the binding is stronger? We have measured the binding of Dys-246 to F-actin in both Tris and Hepes buffers, over a concentration range covering the EM experiments, using a high-speed sedimentation assay (Fig. 4). It can be seen that there is no significant change in the binding curves between the Tris and Hepes buffers, and that these curves are both in excellent agreement with the full binding isotherms determined by Renley et al. (1998). The combined data in Fig. 3 and Fig. 4 therefore show that under conditions where the intrinsic affinity of Dys-246 is remaining relatively constant, the mode of binding to F-actin can change from binding isolated filaments to bundling F-actin.

DISCUSSION

Although the effect of the change from Tris to Hepes (or Mops or Pipes) buffer could be on either F-actin or Dys-246, or both, the erythrosin modification, the tryptic cleav-

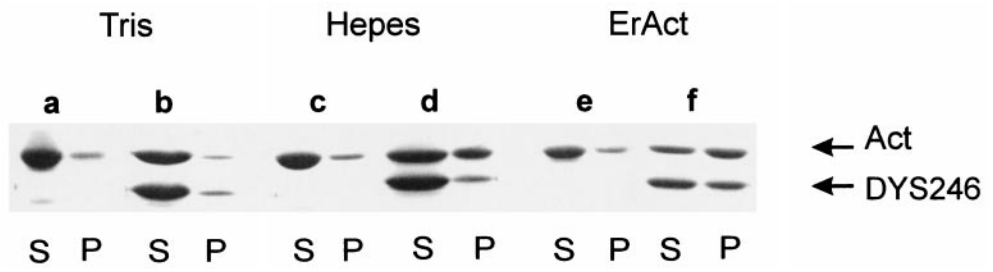

FIGURE 3 Low-speed cosedimentation of Dys-246 with different actins. Coomassie blue-stained SDS gels are shown of supernatants (S) and pellets (P) after centrifugation at 20,000 × *g* for 20 min of 6 μM skeletal muscle actin alone (*a*, *c*, *e*) and with 6 μM Dys-246 (*b*, *d*) in 10 mM Tris (*a*, *b*) or Hepes (*c*, *d*) buffer, 0.1 M KCl, pH 7.6. The experiments with erythrosin-modified skeletal muscle actin (ErAct) (*e*, *f*) were done in Tris buffer, with both proteins present at 3 μM. Similar, very small amounts of Dys-246 pelleted in the absence of actin at low speed in both Hepes and Tris.

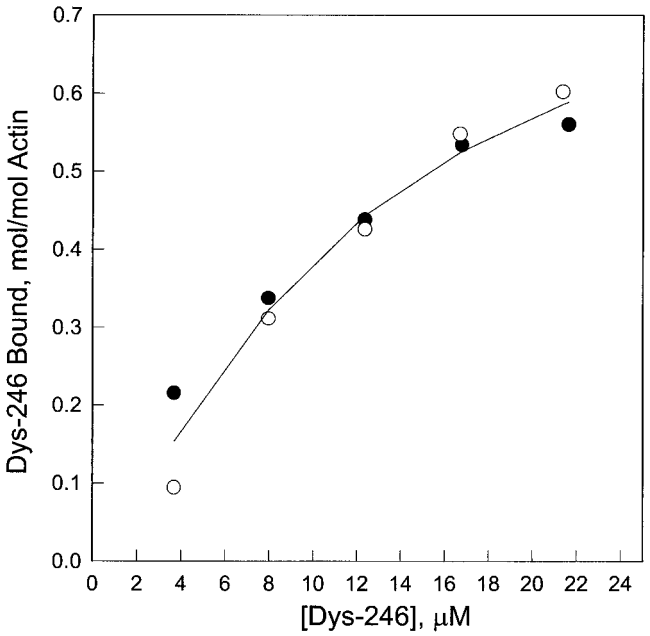

FIGURE 4 A high-speed (100,000 × *g*) sedimentation experiment shows that over the concentration range used for EM experiments, the binding of Dys-246 to actin in Tris (*solid circles*) and Hepes (*open circles*) buffers is nearly indistinguishable. No difference was seen in the amount of Dys-246 that pelleted in the absence of actin between Tris and Hepes. Thus, the change in the mode of binding, observed by both EM (Fig. 1) and low-speed sedimentation (Fig. 3) cannot be explained by a large change in the affinity of Dys-246 for actin.

age, and the 4Ac mutation can only be changing the structure of F-actin. Previous observations have suggested that actin's structure could be modified by trinitrophenylation of lysines using a sulfonic acid derivative (Muhlrad, 1968), and Hepes, Mops, and Pipes contain a related aminosulfonate. It has been directly shown that the aminosulfonate present in Hepes can change the properties of the gap junction protein connexin (Bevans and Harris, 1999). We have used two other sulfonic acid derivative buffers, Mops and Pipes, and observed the same results: skeletal muscle actin and wild-type yeast actin polymerized in these buffers formed bundles in the presence of the fragment. A reconstruction of F-actin in Hepes has failed to detect any significant conformational change at low resolution (Orlova and Egelman, unpublished results), while significant conformational changes were detected after erythrosin modification and tryptic cleavage (Orlova et al., 1995). Thus, there may be a number of different conformational states that allow for Dys-246 to cross-link actin. The conformational change in actin that allows this cross-linking to occur may be very small, and not seen at low resolution.

An interesting possibility is that this conformational change allows for a dimerization of Dys-246 to occur, so that the cross-link formed between two actin filaments involves a dimer of Dys-246. This would be consistent with the observed failure to observe a dimerization of amino-terminal fragments alone (Chan and Kunkel, 1997). However, a more recent report has observed dimers of an amino-terminal fragment of dystrophin both in crystals and in solution (Norwood et al., 2000), suggesting that dimerization of this fragment may be very sensitive to conditions. A dimer of Dys-246 would also be consistent with the span of the cross-link between two actin filaments (Fig. 2 *a*). A single Dys-246 molecule, based upon homology to fimbrin (Puius et al., 1998), would be expected to be fairly compact. Although it is difficult to quantify the cross-bridge size given the disorder and the inability to average, the mass of the cross-bridge seen between two actin filaments (Fig. 2 *a*) appears too large for a single 27-kDa molecule. An actin subunit within a filament is 42 kDa, and the cross-bridges appear to be larger than this. The crystal structure of an equivalent tandem calponin homology domain from utrophin reveals a head-to-tail dimerization (Keep et al., 1999), but the structure also suggests a great conformational plasticity that might give rise to polymorphic interactions with actin. Such plasticity has been invoked in trying to model the stoichiometric binding of the actin-binding domain from utrophin to F-actin (Moores et al., 2000). They suggested that highly related proteins in the fimbrin-utrophin-dystrophin family might have very diverse mechanisms of actin binding based upon rearrangements of the CH domains. Our results suggest that an even greater degree of diversity may arise when actin's structure is modulated by many different means. Although Moores et al. (2000) suggested an induced-fit mechanism, in which utrophin induces a conformational change within the actin filament, and association with the actin filament stabilizes a different conformation of utrophin's actin-binding domain, our observations suggest that different conformations of F-actin may modulate the ability of Dys-246 to cross-link actin.

CONCLUSIONS

What are the implications for these in vitro results, since we do not believe that the cell modifies F-actin by any of the means discussed? It has previously been shown that the nucleation of actin filaments by the actin-binding protein gelsolin induces a long-range conformational change in the actin filament (Orlova et al., 1995; Prochniewicz et al., 1996; Suzuki and Ito, 1996; Khaitlina and Hinssen, 1997; Ressad et al., 1998). Since there is good reason to believe that all actin filaments in the cell are nucleated by other proteins, many in association with membranes or organelles, it is possible that the cell can control by the state of individual actin filaments by the use of different nucleation proteins. An interesting puzzle has been that dystrophin is localized to the sarcolemma, possibly through a direct interaction with actin, and is not found associated with skeletal muscle actin, while the in vitro affinities of Dys-246 are similar for both muscle and nonmuscle iso-

forms of actin (Renley et al., 1998). Our results suggest that the different binding modes of dystrophin may be regulated more by the structural state of the actin filament than by the isoform involved.

Most importantly, the ability of F-actin to exist in multiple conformational states complicates in vitro studies of the interaction between actin and other proteins. The question of what structural states of F-actin exist in vivo remains to be answered, and whether the cell modulates these states to control the interactions with other proteins is an interesting area of speculation.

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