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A Cluster of Basic Repeats in the Dystrophin Rod Domain Binds F-actin through an Electrostatic Interaction*

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The dystrophin rod domain is composed of 24 spectrin-like repeats and was thought to act mainly as a flexible spacer between the amino-terminal actin binding domain and carboxyl-terminal membrane-associated domains. We previously demonstrated that a fragment of the dystrophin rod domain also binds F-actin. However, the nature and extent of rod domain association with F-actin is presently unclear. To begin addressing these questions, we characterized two recombinant proteins representing adjacent regions of the dystrophin rod. DYS1416 (amino acids 1416–1880) bound F-actin with a K_d of 14.2 ± 5.2 μM and a stoichiometry of 1 mol:mol of actin. However, DYS1030 (amino acids 1030–1494) failed to bind F-actin, suggesting that not all rod domain repeats are capable of binding F-actin. Interestingly, DYS1416 corresponds to a unique region of the dystrophin rod rich in basic amino acids, whereas DYS1030 is composed mainly of acidic repeats. This observation suggested that DYS1416 may interact with acidic actin filaments through an electrostatic interaction. Supporting this hypothesis, actin binding by DYS1416 was dramatically inhibited by increasing ionic strength. We suggest that electrostatic interactions between basic spectrin-like repeats and actin filaments may contribute to the actin binding activity of other members of the actin cross-linking protein family.

The skeletal muscle dystrophin-glycoprotein complex is believed to serve as a mechanical link between the actin-based cortical cytoskeleton and merosin in the extracellular matrix (1). Disruption of this linear assembly of proteins due to genetic mutation of dystrophin or its associated glycoproteins results in a variety of muscular dystrophies (2, 3). The actin binding activity of dystrophin had previously been ascribed to a pair of tandem calponin homology domains in the dystrophin amino terminus, which is separated from the membrane-associated cysteine-rich and carboxyl-terminal domains by 24 spectrin-like repeats making up the rod-shaped domain (4). Tandem calponin homology domains form a functional actin binding unit in a number of related proteins, including α-actinin, β-spectrin, and fimbrin (5). Although actin binding by the isolated dystrophin amino terminus had been observed (6–9), it was difficult to reconcile the low affinity of the isolated domain (7, 9) with a potential physiological role for dystrophin-actin binding. We identified a second actin binding site near the middle of the dystrophin rod and demonstrated that the intact dystrophin-glycoprotein complex binds actin with significantly higher affinity than does the isolated amino terminus, presumably through the combined effect of two or more distinct sites (10). These findings suggested an extended, lateral association between dystrophin and the actin filament. Consistent with this model, intact dystrophin, but not its individual actin binding fragments, was found to protect actin filaments from depolymerization (11).

It remains important to determine exactly how much of the very large (314 kDa) dystrophin rod domain is directly involved in binding F-actin. The crystal structure of a stereotypical spectrin α-helical repeat domain yields a length dimension of 5 nm (12), whereas the length contributed by each actin monomer in a filament is 5.5 nm (13). Therefore, each of the 24 spectrin-like repeats of the rod domain could contribute to actin binding by weakly interacting with individual monomers in a single strand of an actin filament. Alternatively, discrete subdomains of the dystrophin rod may specifically bind F-actin, with the remaining repeats acting as spacer regions or as sites for additional cytoskeletal interactions. To begin to address this issue, we have examined the structure and actin binding properties of recombinant proteins encoding fragments of the rod domain lying either amino-terminal (DYS1030) or carboxyl-terminal (DYS1416) to the epitope (amino acids 1416–1494) recognized by dystrophin monoclonal antibody XIXC2 (14). DYS1416 was characterized because it best represented the actin binding proteolytic fragment of dystrophin recognized by XIXC2 (10), whereas DYS1030 represented an adjacent region of the rod domain of comparable length. We have found that actin binding activity is exhibited by a limited portion of the dystrophin rod. Interestingly, the actin binding region of the rod domain overlaps with a cluster of basic repeats in the generally acidic dystrophin rod domain. This, combined with our observation that the actin binding activity of the rod domain is highly sensitive to ionic strength, suggests that a cluster of basic repeats in the dystrophin rod domain binds actin through predominantly electrostatic interactions.

EXPERIMENTAL PROCEDURES

Proteins—Actin was prepared from rabbit skeletal muscle using the method of Pardee and Spudich (15) and further purified by gel filtration on a Sephacryl S-200 column as described previously (10). The construction of pDYS246, encoding dystrophin amino acids 1–246, and pDYS1416, encoding dystrophin amino acids 1416–1880, was previously described (10). A DNA fragment encoding amino acids 1030–1494 of dystrophin was amplified by the polymerase chain reaction from the full-length human dystrophin cDNA (pRSVDY) using the following pair of oligonucleotide primers (Life Technologies, Inc.): 5′-ATCGGATCCTTCCACACTCTTTGTTTCCA-A-3′ and 5′-ATCCATATGAAGAAGCTCTCCTCCCAG-3′. The polymerase

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chain reaction product was subcloned into the TA site of PCRII using the Original TA Cloning kit (Invitrogen). The insert was then cut out with *Nde*I and *Bam*HI and ligated into pET16b previously digested with *Nde*I and *Bam*HI, producing the plasmid pDYS1030. *Escherichia coli* JM109 (DE3) transformed with the dystrophin-encoding plasmids were grown at 37 °C until A_{600} reached 0.8. Cells were then grown an additional 3 h in the presence of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, washed once with 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and frozen. Recombinant proteins were purified using Ni^{2+} affinity chromatography (Novagen) as per the manufacturer's instructions. Eluted proteins were then dialyzed extensively against 20 mM sodium phosphate, pH 8.0, 100 mM (DYS1030 and DYS1416) or 500 mM (DYS246) NaCl or against 10 mM Tris-HCl, pH 8.0, 100 μM dithiothreitol, 100 mM (DYS1030 and DYS1416) or 500 mM (DYS246) NaCl. Recombinant proteins were concentrated in a Centriplus 10 (Amicon). Concentrated proteins were quantitated by A_{280} using native molar extinction coefficients calculated from predicted denatured extinction coefficients and an empirically derived $\epsilon_{nat}/\epsilon_{den}$ ratio (16). After the further addition of dithiothreitol to a concentration of 1 mM and NaN_3 to 0.02%, all recombinant proteins were stored at 4 °C and used within 2 weeks.

F-actin Cosedimentation Assay—F-actin cosedimentation was performed as described previously (10) with the exception that recombinant proteins were subjected to a 20 min, 100,000 × *g* centrifugation step prior to use, in order to remove any protein aggregates. Binding data were analyzed by fitting to the Michaelis-Menten equation using the curve fit function of SigmaPlot.

Circular Dichroism—Purified recombinant proteins in 20 mM sodium phosphate, pH 8.0, and varying NaCl concentrations (100–800 mM) were analyzed by circular dichroism at 25 °C in an Aviv 62A DS circular dichroism spectrometer with a path length of 1 mm. Samples from at least two different preparations were analyzed at concentrations ranging from 0.2 to 30 μM, and the molar residue ellipticity spectra were calculated. Percentage of α-helical content was estimated by assuming that a molar residue ellipticity of −36,000 degrees cm^2 $dmol^{-1}$ at 222 nm corresponded to 100% α-helical content (17).

Sequence Analysis—The predicted amino acid sequences of proteins were analyzed using the computer programs Isoelectric and Peptidesort of the Genetics Computer Group. Domain and repeat boundaries were adopted from Winder *et al*. (18).

RESULTS

In order to begin addressing the question of what portion of the large dystrophin rod domain is directly involved in binding actin filaments, we produced recombinant proteins encoding dystrophin amino acids 1030–1494, containing repeats 7–10 (DYS1030), and amino acids 1416–1880, containing repeats 11–14 (DYS1416). A third recombinant protein (DYS246), encoding residues 1–246 of the well characterized dystrophin amino-terminal domain, served as an actin binding control (Fig. 1). In order to verify that the recombinant proteins had assumed appropriately folded structures, we analyzed each by circular dichroism. Each of the three proteins exhibited the characteristic double-minimum spectrum indicative of high α-helical content (Fig. 2). The α-helical content of each protein (Table I) was calculated from the average ($n \geq 2$) molar residue ellipticity at 222 nm (17). The value of 39% α-helix for DYS246 is consistent with a value of 42% reported for the amino-terminal domain of utrophin (19). The α-helical contents for both DYS1030 (54%) and DYS1416 (70%) are consistent with independent biophysical studies of the dystrophin rod domain (20–22). Although DYS1030 exhibited a lower α-helical content than did DYS1416, a recombinant protein (RFM) spanning nearly 90% of the combined sequence encoded by DYS1030 and DYS1416 previously exhibited an α-helical content of 62% (Table I). Because this value is midway between the values that we measured for DYS1030 and DYS1416, we conclude that the lower value for DYS1030 was not due to improper folding of the protein but rather to an intrinsically lower helical content for the portion of the rod domain represented by DYS1030. We recorded circular dichroism spectra for each protein over a range of NaCl concentrations from 100 to 800 mM and found no significant qualitative or quantitative salt-dependent changes in the spectra (not shown). We therefore conclude that each recombinant dystrophin fragment assumed a stable, highly α-helical structure.

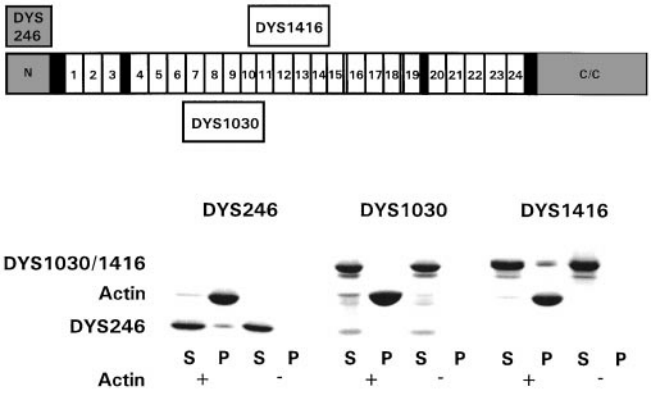

FIG. 1. **Cosedimentation of recombinant dystrophin fragments with F-actin.** Shown in the *upper panel* is a schematic representation of dystrophin, illustrating its amino-terminal domain (*N*) and cysteine-rich and carboxyl-terminal (*C*/*C*) domains, as well as the 24 spectrin-like repeats making up the rod-shaped domain. Putative hinge regions are *shaded* in *black*. Also shown are the relative locations of the recombinant proteins DYS246 (amino acids 1–246), DYS1030 (1030–1494), and DYS1416 (1416–1880). The *lower panel* shows Coomassie Blue-stained SDS-polyacrylamide gels of supernatants (*S*) and pellets (*P*) following a 30-min incubation and 100,000 × *g* centrifugation of 5 μM recombinant dystrophin fragments in the presence (+) or absence (−) of 5 μM F-actin.

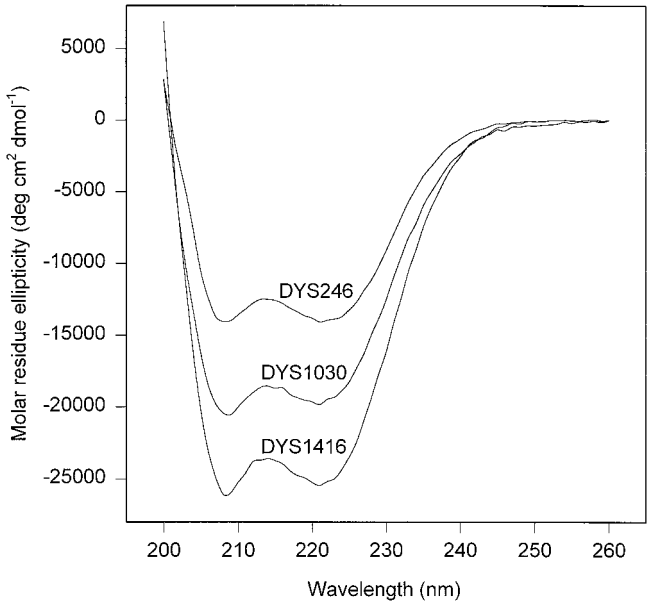

FIG. 2. **Circular dichroism analysis of recombinant dystrophin fragments.** Purified recombinant dystrophin fragments were analyzed by circular dichroism at 25 °C in an Aviv 62A DS circular dichroism spectrometer with a path length of 1 mm. Shown is the molar residue ellipticity as a function of wavelength, averaged from experiments carried out at various protein concentrations. No significant qualitative or quantitative changes in circular dichroism spectra were observed over a range of NaCl concentrations from 100 to 800 mM.

Using a high-speed cosedimentation assay, we tested the ability of each recombinant protein to bind F-actin. Consistent with our previous observations, DYS246 and DYS1416 specifically cosedimented with F-actin (Fig. 1). Saturable binding was reproducibly achieved by the cosedimentation of increasing concentrations of DYS1416 with a fixed amount of F-actin. Fitting the binding data from three independent experiments to the Michaelis-Menten equation showed that DYS1416 bound F-actin with a K_d of 14.2 ± 5.2 μM, a B_{max} of 1.17 ± 0.5, and a Hill coefficient of 1.64 ± 0.4 (Fig. 3).

TABLE I
Characterization of recombinant dystrophin fragments by circular dichroism

Recombinant protein	Dystrophin amino acids	Molar ellipticity at 222 nm	α-Helical content	Ref.
		degrees cm^2 $dmol^{-1}$	*%*	
DYS246	1–246	−14,000	39	This study
DYS1030	1030–1494	−19,500	54	This study
DYS1416	1416–1880	−25,000	70	This study
RFM	1141–1973	−22,200	62	22

Scatchard analysis yielded very similar values of K_d = 22 μM and B_{max} = 1.25. These values are similar to those measured for recombinant proteins corresponding to the amino-terminal domain of dystrophin, including DYS246 (7, 9, 23). We also tested the ability of DYS246 and DYS1416 to competitively inhibit intact dystrophin binding to F-actin. Although the binding of 0.2 μM dystrophin-glycoprotein complex to 5 μM F-actin was reduced by 76 or 55% in the presence of 30 μM DYS246 or DYS1416, respectively, neither recombinant protein competed with the other for binding to F-actin at concentrations as high as 85 μM (not shown). These results indicated that although DYS246 and DYS1416 bind F-actin with similar affinities and stoichiometries, they likely interact with distinct sites on the actin filament. In contrast to our observations of DYS246 and DYS1416, DYS1030 reproducibly failed to bind F-actin (Fig. 1). This result is consistent with our previous finding that a proteolytic dystrophin fragment approximately corresponding to DYS1030 did not cosediment with F-actin (10). These observations indicated that actin binding activity is limited to a subset of the spectrin-like repeats making up the dystrophin rod domain.

Based on our finding that not all dystrophin rod domain repeats are capable of binding F-actin, it remained necessary to identify the unique structural features that confer actin binding activity. Interestingly, the program Peptidesort indicated that the predicted pI of the actin binding recombinant protein DYS1416 was 8.0, whereas the pI of nonbinding DYS1030 was 5.1 (Fig. 4*A*). The calculated pI value for each recombinant protein was verified (not shown) by isoelectric focusing (DYS246 and DYS1030) or by nonequilibrium pH gradient electrophoresis (DYS1416). Because of the notable difference between the pI of DYS1030 and DYS1416, we subdivided the amino acid sequence of the dystrophin rod domain into its 24 constitutive spectrin-like repeats (18), calculated the predicted pI of each individual repeat, and plotted the results as a function of repeat number (Fig. 4*B*). Interestingly, we found that DYS1416 overlaps with a cluster of basic repeats (repeats 11–17) in the otherwise acidic dystrophin rod. In fact, three of the four repeats making up DYS1416 are basic, whereas DYS1030 contains only one basic repeat. These analyses revealed that DYS1030 and DYS1416 are distinguished by a significant difference in net charge and suggested that the excess of positive charges on DYS1416 may play a role in its specific interaction with the negatively charged actin filament. In support of the significance of these findings, the organization of acidic and basic repeats is conserved between human and mouse dystrophins (not shown).

Electrostatic protein-protein interactions are distinguished by a dramatic sensitivity to ionic strength (24). Therefore, in order to test whether the binding of DYS1416 to actin is due predominantly to electrostatic interactions, we measured the NaCl sensitivity of actin binding by DYS246 and DYS1416. We subjected DYS246 and DYS1416 to high-speed F-actin cosedimentation in actin binding buffer containing a range of NaCl concentrations from 100 to 800 mM (Fig. 5). Actin binding by

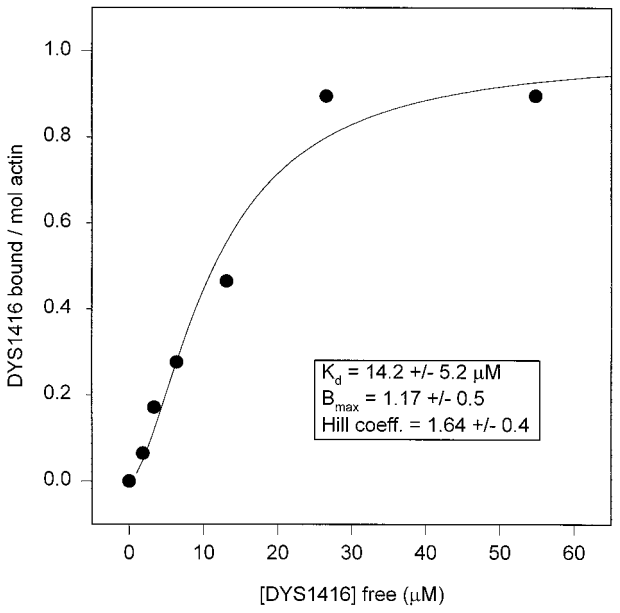

FIG. 3. **DYS1416 binding to F-actin.** Increasing concentrations of DYS1416 were incubated with 5 μM F-actin and centrifuged at 100,000 × *g*. The amount of free and bound DYS1416 was determined densitometrically from Coomassie Blue-stained gels of supernatant and pellet fractions as shown in Fig. 1. Binding data fitted by nonlinear regression analysis are shown by the *line*. *Symbols* represent the average of three independent experiments.

DYS1416 was found to be highly sensitive to increasing ionic strength, exhibiting an IC_{50} of approximately 200 mM NaCl, with binding essentially abolished at 400 mM. In contrast, actin binding by DYS246, which is generally thought to occur primarily through hydrophobic interactions (6), was largely retained at high NaCl concentration (85 and 75% of control at 200 and 400 mM, respectively). Because no detectable changes in the circular dichroism spectrum of DYS1416 or the fraction of F-actin pelleted were observed over the range of NaCl concentrations tested, we conclude that a neutralization of attractive electrostatic forces between basic repeats and the actin filament is responsible for the observed inhibition, rather than a large-scale conformational change in either protein. The strong ionic strength sensitivity of DYS1416 binding to F-actin and the relatively insensitive binding of DYS246 are also consistent with the intermediate ionic strength sensitivity of F-actin binding by the dystrophin-glycoprotein complex (10). Collectively, the data presented here indicate that two distinct actin binding sites on dystrophin contribute to the binding properties of the native molecule and, furthermore, that electrostatic interactions between actin filaments and a cluster of basic repeats in the dystrophin rod are responsible for the rod domain actin binding activity.

DISCUSSION

The function of the large (314 kDa) rod domain of dystrophin has long been unresolved. The 24 spectrin-like repeats that make up the rod were hypothesized (4, 25, 26) to play a role in dystrophin dimerization, based on analogy to its sequence relatives, α-actinin and β-spectrin. However, the predictive capabilities of such comparisons are limited, as attested to by our (11) and others' (22, 27) observations that dystrophin exists in a monomeric state. The relatively low sequence conservation between dystrophin repeats also argued against rod domain dimerization and led to an alternative hypothesis for the function of the dystrophin rod domain (18, 28). In addition to serving as an extended, flexible spacer between the actin bind-

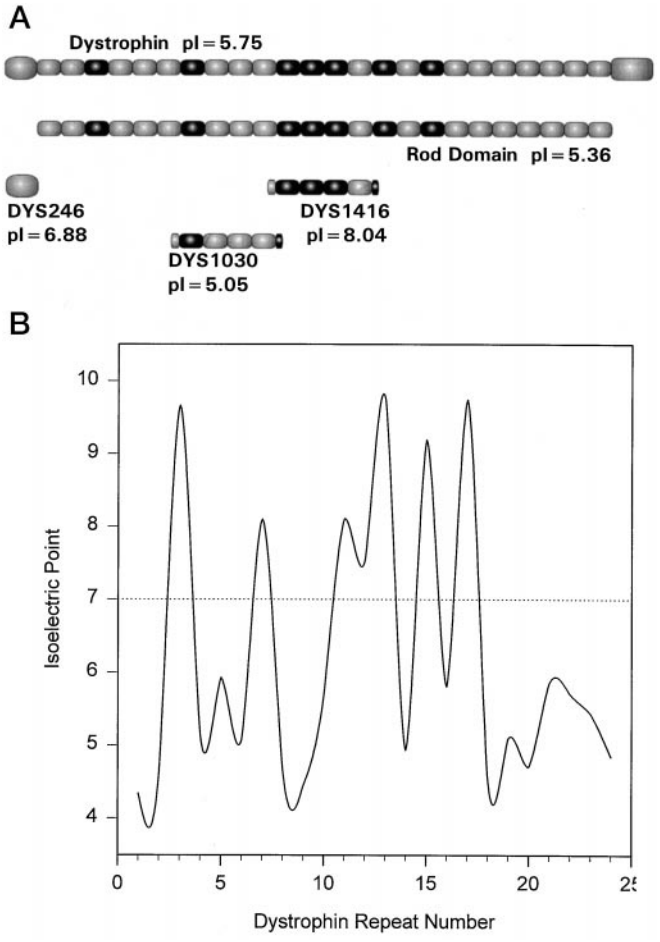

FIG. 4. **Predicted isoelectric points of dystrophin and its constitutive domains.** Shown in *A* are schematic representations of either full-length dystrophin or fragments of dystrophin and the predicted isoelectric point of each protein. Basic repeats are shown *shaded* in *black*. *B,* the amino acid sequence of human dystrophin was subdivided into its 24 constitutive α-spectrin-like repeats (18). The predicted isoelectric point of each repeat was then calculated using the computer program Peptidesort (Genetics Computer Group) and plotted *versus* the repeat number.

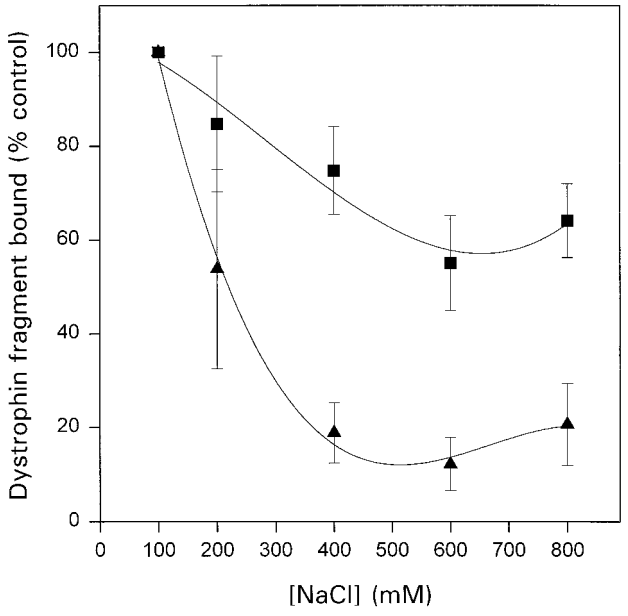

FIG. 5. **NaCl sensitivity of DYS246 and DYS1416 binding to F-actin.** 5 μM of either DYS246 (*squares*) or DYS1416 (*triangles*) was incubated with an equimolar concentration of F-actin in actin binding buffer containing a range of NaCl concentrations (100–800 mM) and subjected to cosedimentation analysis as described in Fig. 1. Binding data ($n \geq 5$) are expressed as percentage of binding measured in 100 mM NaCl.

ing amino terminus and the membrane-associated carboxyl terminus, it was suggested that the rod domain may also contain sites for additional cytoskeletal interactions. Our discovery (10) of a novel actin binding activity located within residues 1416–1880 of dystrophin provided the first direct evidence for the latter possibility. We showed that both the amino terminus and the central rod domain interact with actin filaments and, furthermore, that the two spatially separated binding sites on a single dystrophin molecule induce a lateral association with the actin filament, providing *in vitro* stabilization against actin depolymerization (11). Interestingly, a recombinant protein corresponding to the second, acidic spectrin-like repeat of dystrophin was observed to interact specifically with artificial membranes containing phosphatidylserine (29). This observation may provide a partial basis for the targeting of dystrophin to the sarcolemma and reinforces the idea that the rod domain likely plays a much more significant and complex role in dystrophin function than was formerly appreciated.

Although the identification of a novel actin binding site in the dystrophin rod domain resulted in a significant revision of the role of dystrophin in anchoring the cortical cytoskeleton to the sarcolemma, it remained important to determine the nature and extent of the interaction between actin and the dystrophin rod. One outstanding question was whether actin binding is a general feature of individual spectrin-like repeats making up the rod domain or whether actin binding activity is restricted to a subset of the repeats. Our current observations indicate that only a limited portion of the dystrophin rod domain exhibits actin binding activity. Furthermore, this activity is likely to be due to electrostatic interactions between the acidic actin filament and a cluster of basic repeats in the dystrophin rod.

That rod domain actin binding activity is restricted to a region rich in basic residues is consistent with results obtained for a variety of actin-binding proteins (30–32). For example, Tang *et al.* (33) observed that the effects of the highly basic protein calponin on actin polymerization and bundling were due primarily to its ability to reduce the electrostatic repulsion between polyanionic actin filaments. In contrast, intact dystrophin has no effect on actin polymerization (11) and does not cross-link/bundle F-actin (10). Thus, whereas the cluster of basic repeats appears to function as a polycationic ligand for F-actin, the extensive acidic repeat regions flanking the cluster of basic repeats (Fig. 4) may provide sufficient interfilament repulsion to prevent filament bundling (10). Furthermore, the basic myristoylated alanine-rich protein kinase C substrate-related domain of adducin is critical to its actin binding activity (31). Interestingly, the cluster of basic residues in MARCKS has been implicated in its binding to both actin (32) and anionic phospholipids (34), which raises the possibility that the basic repeats of the dystrophin rod domain may dually serve to bind actin and retain dystrophin near the sarcolemmal membrane.

Our observation that basic spectrin-like repeats contribute to the actin binding activity of a tandem calponin homology domain-containing protein raises the question of whether this may be a property common to sequence relatives of dystrophin. Whereas the amino-terminal domain of β-spectrin was observed to bind F-actin with 26 μM affinity (35), the addition of its first repeat increased the affinity for F-actin by 4-fold and decreased the stoichiometry by a factor of 3. The sequential addition of up to nine more repeats had no effect on the affinity

for actin, although the second repeat appeared to play a role in binding additional cytoskeletal elements. Interestingly, the first β-spectrin repeat has a calculated pI of 10.1, but repeats 2–10 are all acidic, with pI values ranging from 4.2 to 6.7. Thus, it appears that the presence of even a single basic repeat immediately adjacent to tandem calponin homology domains can have a significant effect on actin binding affinity. It is also possible that the more modest differences between the actin binding affinities of native α-actinin and its recombinant amino terminus (Ref. 36 and references therein) can be explained by the presence of a single basic repeat (pI = 8.1) immediately adjacent to its amino-terminal domain. Supporting this possibility, F-actin binding by the isolated amino-terminal domain of α-actinin was relatively insensitive to increasing ionic strength (24), as compared with the marked inhibition of the native molecule (37). Thus, as is the case for dystrophin, electrostatic interactions may contribute to the actin binding activity of other proteins in the spectrin superfamily.

A number of recent studies (38–40) have suggested that dystrophin may be functionally interchangeable with its autosomal homolog, utrophin. Indeed, the isolated amino terminus of utrophin bound F-actin with characteristics similar to the dystrophin amino terminus (9), and the overexpression of a utrophin minigene resulted in substantial but incomplete correction of the dystrophic phenotype of *mdx* mice (39, 41, 42). Based on our preliminary findings for the dystrophin rod domain and assuming that utrophin is functionally homologous with dystrophin, we expected to identify a similar cluster of basic repeats in the utrophin rod domain that would enable it to also bind F-actin. Surprisingly, a plot of the calculated pI of individual human utrophin rod domain repeats revealed that only repeats 4 and 21 of utrophin are basic (pI = 7.5 and 8.4, respectively), with the remaining 20 repeats exhibiting calculated pI values ranging from 3.8 to 6.3. As is the case for dystrophin, this pattern of acidic and basic repeats is preserved in mouse utrophin. Because a single isolated basic repeat appears to be incapable of binding F-actin (DYS1030), we hypothesize that utrophin may lack the rod domain actin binding activity that we have identified in dystrophin.

The present data do not rule out the possible existence of a single, more discreet actin contact site within dystrophin amino acids 1416 and 1880. On the other hand, our plot of pI *versus* repeat number (Fig. 4*B*) suggests that repeats 11–17 could form a more extensive electrostatic association with actin. Therefore, in addition to examining the potential roles of spectrin-like repeats in the actin binding activities of α-actinin and utrophin, it will be necessary to more rigorously map the actin binding sites in the dystrophin rod domain.

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