

Utrophin Lacks the Rod Domain Actin Binding Activity of Dystrophin*

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We previously identified a cluster of basic spectrin-like repeats in the dystrophin rod domain that binds F-actin through electrostatic interactions (Amann, K. J., Renley, B. A., and Ervasti, J. M. (1998) *J. Biol. Chem.* 273, 28419–28423). Because of the importance of actin binding to the presumed physiological role of dystrophin, we sought to determine whether the autosomal homologue of dystrophin, utrophin, shared this rod domain actin binding activity. We therefore produced recombinant proteins representing the cluster of basic repeats of the dystrophin rod domain (DYSR11–17) or the homologous region of the utrophin rod domain (UTROR11–16). Although UTROR11–16 is 64% similar and 41% identical to DYSR11–17, UTROR11–16 ($pI = 4.86$) lacks the basic character of the repeats found in DYSR11–17 ($pI = 7.44$). By circular dichroism, gel filtration, and sedimentation velocity analysis, we determined that each purified recombinant protein had adopted a stable, predominantly α -helical fold and existed as a highly soluble monomer. DYSR11–17 bound F-actin with an apparent K_d of $7.3 \pm 1.3 \mu M$ and a molar stoichiometry of 1:5. Significantly, UTROR11–16 failed to bind F-actin at concentrations as high as $100 \mu M$. We present these findings as further support for the electrostatic nature of the interaction of the dystrophin rod domain with F-actin and suggest that utrophin interacts with the cytoskeleton in a manner distinct from dystrophin.

Dystrophin is the 427-kDa protein product of the Duchenne muscular dystrophy locus. It binds both F-actin and a complex of membrane-associated proteins that binds merosin, thereby providing a physical connection between the cortical cytoskeleton and the extracellular matrix (2). The importance of this functional complex of proteins to striated muscle physiology is demonstrated by the observations that genetic disruption of dystrophin, merosin, or several other protein components of the dystrophin-glycoprotein complex results in muscular dystrophies.

The dystrophin sequence consists of four distinct domains (Fig. 1): an amino-terminal, calponin homology-type actin bind-

ing domain; a large, rod-shaped domain comprised of 24 spectrin-like repeats and four hinge regions; a cysteine-rich domain and a unique carboxyl-terminal domain (3). Sequences located in the cysteine-rich and carboxyl-terminal domains are responsible for dystrophin binding to the glycoprotein complex and other associated proteins (4–6). Until recently, it was generally thought that dystrophin bound cortical actin solely through its amino-terminal actin binding domain (7–11). However, transgenic mice expressing a dystrophin construct deleted for the actin binding amino-terminal domain presented with a surprisingly mild phenotype, indicating that the amino-terminal domain is not specifically essential for dystrophin function (12). Furthermore, we found that native dystrophin bound F-actin with substantially higher affinity and lower stoichiometry than did the isolated amino-terminal actin binding domain (13). We also identified a novel actin binding activity located between the 11th and 14th spectrin-like repeats in the dystrophin rod domain (13). From these and other data, we proposed that the two independent and physically separated actin binding sites act in concert to effect a high affinity, lateral association between native dystrophin and F-actin.

We have since demonstrated that the interaction of the dystrophin rod domain with F-actin is limited to a subset of the rod domain repeats and that the interaction is due to electrostatic interactions between the acidic actin filament and a unique cluster of basic repeats in the otherwise acidic dystrophin rod domain (1). While our previous studies employed a recombinant protein representing repeats 11–14, sequence analysis (Fig. 1) revealed that the cluster of basic repeats extends from repeat 11 to repeat 17, suggesting that a more extensive lateral association between the dystrophin rod domain and F-actin may be possible.

A number of recent studies (14–20) have suggested that dystrophin may be functionally interchangeable with its autosomal homologue, utrophin. Utrophin is highly expressed throughout fetal and regenerating muscles but is down-regulated at birth and restricted to the myotendinous and neuromuscular junctions of normal adult muscle (21). Because utrophin also displays several of the protein binding activities described for dystrophin, utrophin was hypothesized to function as a fetal homologue of dystrophin that may be capable of compensating for dystrophin deficiency. Indeed, continued utrophin expression in *mdx* mice partially attenuates the phenotype associated with dystrophin deficiency, since mice lacking both dystrophin and utrophin exhibit a more severe phenotype similar to that presented by patients with Duchenne muscular dystrophy (16, 17). In *mdx* mice, transgenic overexpression of full-length utrophin results in further functional improvement (14), while overexpression of a truncated utrophin lacking the middle rod domain resulted in less complete recovery (15, 20). Based on these studies, we expected to identify a cluster of basic, spectrin-like repeats in the utrophin rod

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domain that would enable it to laterally bind F-actin in a manner similar to that shown for dystrophin. Surprisingly, only utrophin repeats 4 and 21 were found to be basic, with the remaining 20 repeats exhibiting calculated pI values ranging from 3.8 to 6.3 (Fig. 1). Although it has been demonstrated that the amino-terminal domain of utrophin binds actin with similar characteristics to that of dystrophin (22), this observation led us to hypothesize that utrophin may lack the rod domain actin binding activity present in dystrophin (1).

To determine whether dystrophin rod domain repeats 11–17 form a more extensive lateral association than do repeats 11–14 and to determine whether utrophin's central rod domain is capable of binding F-actin, we produced recombinant proteins representing the complete cluster of basic repeats of the dystrophin rod (DYSR11–17) or the corresponding region from utrophin (UTROR11–16). By circular dichroism, gel filtration, and sedimentation velocity analysis, we found that each purified recombinant protein adopted a stable, predominantly α -helical fold and existed as a highly soluble monomer over a wide range of concentrations and buffer conditions. DYSR11–17 bound F-actin with 7.3 μ M affinity and 1:5 molar stoichiometry, while UTROR11–16 failed to bind actin at concentrations as high as 100 μ M. We presently conclude that the entire cluster of basic repeats, spanning repeats 11–17 of the dystrophin rod domain, form a higher affinity and more extensive lateral association with F-actin than previously demonstrated with a subset of the cluster. Furthermore, the failure of the homologous, but acidic, utrophin middle rod domain to bind actin is further support for the electrostatic nature of the interaction of the basic dystrophin repeats with actin and suggests that utrophin interacts with the cytoskeleton in a manner distinct from dystrophin.

EXPERIMENTAL PROCEDURES

Sequence Analysis—The predicted amino acid sequences of proteins were analyzed using the Genetics Computer Group software package. Domain and repeat boundaries were adopted from Winder *et al.* (23).

Recombinant Proteins—A DNA fragment encoding rod domain repeats 11–17 (amino acids 1461–2209; Fig. 1) of human dystrophin, beginning with the first amino acid of repeat 11 immediately following an initiation methionine and terminating immediately after the final residue of repeat 17, was amplified from pRSVDy (24) by the polymerase chain reaction using the following pair of oligonucleotide primers: 5'-GGGAATTCATATGTTCCAGAAACCAGCCAAT TTTGAGC-3' and 5'-ATAAGAATGCGGCCGCTATTGTTCTTCTAGCCTCTTTTCTGTC-3'. The resulting DNA fragment was subcloned into the *Sma*I site of pUC18, excised with *Nde*I and *Not*I, and ligated into the *Nde*I/*Not*I site of pET23a to produce the plasmid pDYSR11–17. Similarly, a DNA fragment encoding rod domain repeats 11–16 (amino acids 1433–2081, Fig. 1) of mouse utrophin, beginning with the first amino acid of repeat 11 and terminating immediately after the final residue of repeat 16, was amplified from the cDNA (25) using the following pair of oligonucleotide primers: 5'-GCATGCAGTCCATATGTTCCAGAAAGCCCGCAA-ATTTGAG-3' and 5'-GCATGCAGTCCGCGGCCGCTCATTCTCTTTT-TAGCCTGGGCTGCAA-3'. The resulting product was digested with *Not*I and *Nde*I and ligated into the *Not*I/*Nde*I site of pET23a, producing the plasmid pUTROR11–16. JM109 (DE3) *Escherichia coli* transformed with either pDYSR11–17 or pUTROR11–16 were grown and induced under standard conditions (1). Frozen DYSR11–17 *E. coli* pellets were resuspended in 6 M urea, 10 mM Tris-HCl, pH 8.5, 5 mM DTT¹ with sonication. Supernatants following a 20-min, 49,000 \times g centrifugation were filtered through two layers of Miracloth and applied to an SP Sepharose column pre-equilibrated with the same buffer. Bound proteins were eluted with a linear 0–250 mM NaCl gradient. As in all purification steps, fractions were analyzed on Coomassie Blue-stained SDS-polyacrylamide gels. Peak fractions were pooled and refolded by dialysis against 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM DTT. After concentration in a Centrplus 50, the sample was applied to an Sephacryl S-200 column and eluted in the same buffer. Peak fractions

were then diluted to 40 mM NaCl with Q buffer (10 mM Tris-HCl, pH 7.4, 5 mM DTT) and applied to a DEAE column. The DEAE void was applied to a Q Sepharose column and eluted with a linear NaCl gradient in Q buffer. Peak fractions were dialyzed against 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT and again concentrated in a Centrplus 50 concentrator. Protein concentration was determined as described previously (1). UTROR11–16 was purified by similar methods, except for the following modifications. Frozen UTROR11–16 pellets were resuspended in 6 M urea, 10 mM Tris-HCl, pH 6.0, 5 mM DTT with sonication. The filtered 49,000 \times g supernatant was applied to a Q Sepharose column and eluted with a linear NaCl gradient. Peak fractions were refolded by dialysis against 200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM DTT. Following concentration in a Centrplus 50 concentrator, the sample was applied to a S-200 column and eluted with the same buffer. Peak fractions were dialyzed against 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT, concentrated, and quantitated as described above.

Circular Dichroism—Purified recombinant proteins were analyzed by circular dichroism spectroscopy in a Aviv 62A DS circular dichroism spectrometer with a path length of 1 mm. Samples were analyzed at 25 °C at concentrations ranging from 1 to 10 μ M. Percentage α -helical content was calculated by assuming a molar residue ellipticity of $-36,000$ degrees $\text{cm}^2 \text{dmol}^{-1}$ at 222 nm for corresponding to 100% α -helix (26). NaCl concentration was varied from 50 to 600 mM. Urea concentration was varied from 0 to 3 M. Thermal denaturation was monitored by circular dichroism at 222 nm as temperature was varied in intervals of 5 °C over the range of 25–95 °C.

F-actin Binding Assay—Actin binding assays were performed essentially as described previously (1), using 5 μ M F-actin in all assays. Binding data were adjusted for both the fraction of recombinant protein sedimented in the absence of actin and the amount (3% of total, as measured with a bovine serum albumin control) trapped in the volume of the actin pellet (8).

Sedimentation Velocity—Sedimentation velocity values of recombinant proteins were determined by sucrose gradient ultracentrifugation. Thyroglobulin, catalase, aldolase, and albumin (Amersham Pharmacia Biotech) were used as standards. 100 μ g of each recombinant or standard protein was layered onto 12-ml, linear 5–20% (w/v) sucrose gradients in buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT and centrifuged at 50,000 rpm for 90 min at 10 °C in a 65.1 VTi rotor. 500- μ l fractions were collected and analyzed by SDS-PAGE and Coomassie Blue staining.

Stokes Radius—The Stokes' radii of recombinant proteins were determined by gel filtration chromatography on a 25 \times 425-mm S-200 Sephacryl column, at a flow rate of 2 ml/min in 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM DTT, using aldolase, albumin, chymotrypsinogen A, and RNase A as standards. 2-ml fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.

Data Analysis—F-actin binding data were analyzed by nonlinear regression analysis by fitting to the equation (27),

$$B = [S]^n \times B_{\max} / (K_d^n + [S]^n) \quad (\text{Eq. 1})$$

using the CurveFit function of SigmaPlot. Native molecular weight and frictional coefficients were determined using the equations (28),

$$M_r = (6\pi\eta NR_s) / (1 - \nu\rho_{20,w}) \quad (\text{Eq. 2})$$

and

$$f/f_o = R_s(4\pi N/3\nu M_r)^{1/3} \quad (\text{Eq. 3})$$

where $\eta = 0.010019$ g/s \cdot cm, $N = 6.02 \times 10^{23}$, R_s represents the Stokes' radius (cm), s represents the sedimentation coefficient (Svedbergs), ν represents partial specific volume (g/ml), and $\rho_{20,w} = 0.99823$ g/ml.

RESULTS

Our previous limited proteolysis and recombinant protein studies demonstrated that repeats 11–14 of the dystrophin rod domain were capable of binding actin *in vitro*, while repeats 7–10 lacked actin binding activity (1, 13). Sequence analysis (Fig. 1) revealed that dystrophin repeats 11–14 were basic but that 11–17 might form more extensive lateral association with actin. Also, analysis of the dystrophin homologue utrophin (Fig. 1) indicated that its rod domain lacked the cluster of basic repeats. To further test whether basic character conferred the mid-

¹ The abbreviation used is: DTT, dithiothreitol.

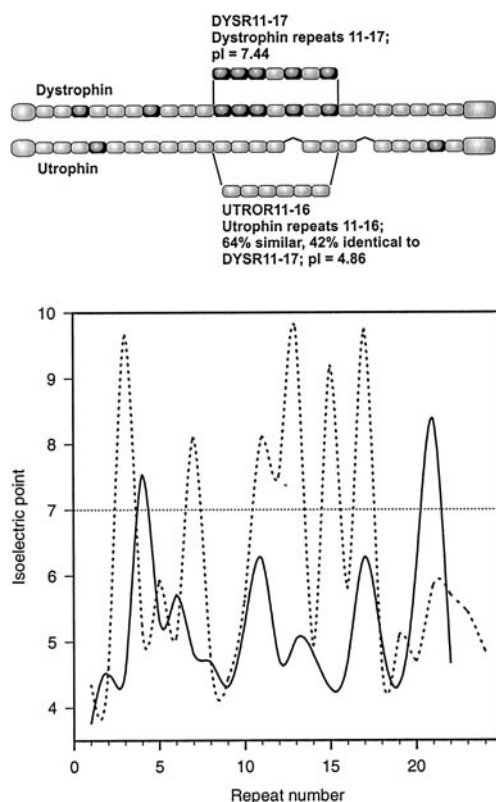


FIG. 1. *A*, a schematic representation of dystrophin, utrophin, and the two recombinant fragments of their respective rod domains. Basic repeats are shaded black. Repeat numbers and predicted isoelectric points of the recombinant proteins are also shown. Note that sequence analysis indicates that utrophin lacks repeats corresponding to repeat 15 and repeat 19 of dystrophin. Hinge regions following the amino-terminal domain, repeat 3, repeat 19, and repeat 24 of dystrophin and corresponding domains of utrophin are not shown. *B*, predicted isoelectric points of dystrophin and utrophin repeats. The amino acid sequence of either utrophin (solid line) or dystrophin (dotted line) was subdivided into its constitutive spectrin-like repeats (23). The predicted isoelectric point of each repeat was then calculated using the computer program Peptidesort (Genetics Computer Group) and plotted versus the repeat number.

dle rod domain actin binding activity, we expressed and characterized recombinant proteins encoding the middle rod domain of dystrophin (DYSR11–17) and utrophin (UTROR11–16).

We first characterized the hydrodynamic properties of each purified recombinant protein. By gel filtration chromatography and sedimentation velocity analysis (Table I), we determined the native molecular weight of each protein as well as its frictional coefficient. The measured native molecular weight of each protein was within 6% of its predicted monomeric molecular weight, therefore indicating that each protein, in addition to being highly soluble under a variety of buffer conditions, existed predominantly as a monomer in solution. These data are further support for the monomeric nature of dystrophin and the first evidence for a monomeric state of utrophin. Additionally, the frictional coefficients of DYSR11–17 (1.86) and UTROR11–16 (1.74) and Stokes' radii (5.50 and 4.75 nm, respectively) indicated that both proteins assumed an asymmetric shape consistent with their predicted rod shapes.

To assess proper folding, we performed circular dichroism analysis of the two proteins and found that, based on the magnitude of ellipticity at 222 nm (26) and the ratio of ellipticities at 209 and 222 nm, each possessed a high degree of α -helical content (Fig. 2*a*, Table I). These findings were consistent with both predictions based on the primary structure of the dystrophin rod domain and previous experimental obser-

TABLE I
Physical properties of rod domain fragments

Properties	DYSR11–17	UTROR11–16
No. of residues	750	650
Predicted M_r	87,899	73,835
Native M_r	87,277	69,966
pI	7.44	4.86
α -Helical content	62%	68%
T_m	55.6 °C	56.5 °C
Stokes radius	5.50 nm	4.75 nm
Sedimentation coefficient	3.70 s	3.42 s
Frictional coefficient	1.86	1.74

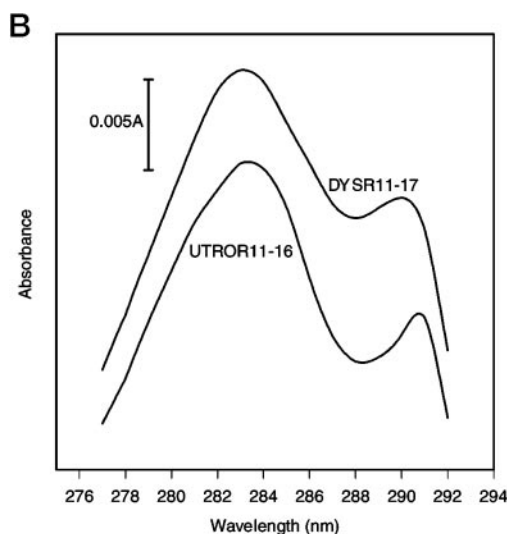
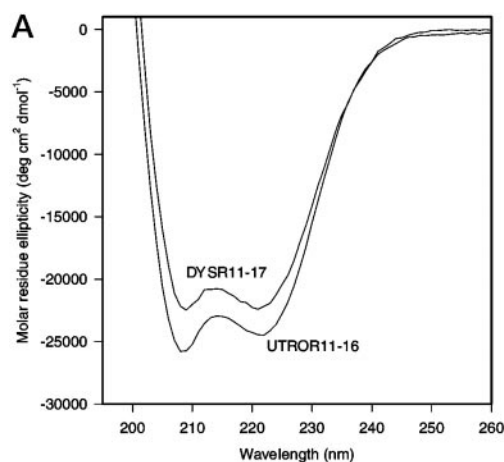


FIG. 2. *A*, circular dichroism spectra of recombinant rod domain fragments. Purified DYSR11–17 or UTROR11–16 was analyzed by circular dichroism at 25 °C in an Aviv 62A DS Circular Dichroism Spectrometer with a path length of 1 mm. Shown is the molar residue ellipticity as a function of wavelength, averaged from two independent experiments each. No significant qualitative or quantitative changes in CD spectra were observed over a wide range of protein (1–10 μ M) or NaCl (50–600 mM) concentrations. *B*, ultraviolet absorbance spectra of rod domain fragments. The absorbance spectrum of DYSR11–17 or UTROR11–16 was obtained at 25 °C in an Aviv 62A DS Circular Dichroism Spectrometer with a path length of 1 mm, using an excitation cut-off of 320 nm and protein concentrations of approximately 1 μ M. Note the prominent shoulder in both spectra at 288–290 nm, indicative of stably folded spectrin-like repeats (32).

vations (1, 29–31) of dystrophin rod domain fragments. We also analyzed the ultraviolet absorbance spectra of DYSR11–17 and UTROR11–16 and observed in each spectrum the presence of a pronounced shoulder between 288 and 290 nm (Fig. 2*B*), which is a strong marker for stably folded dystrophin repeats (32). Additionally, we determined the urea denaturation profiles of

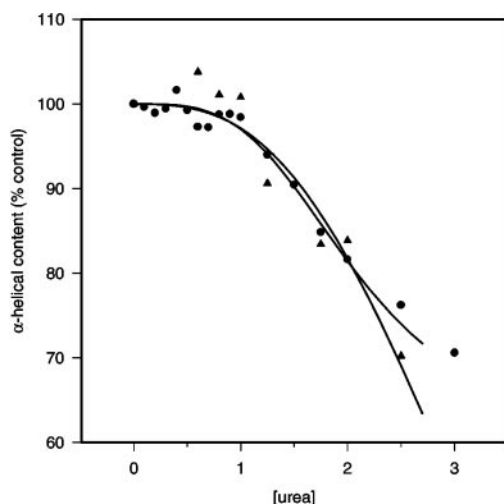


FIG. 3. Resistance of rod domain fragments to denaturation by urea. α -Helical content of DYSR11-17 (circles) or UTROR11-16 (triangles) was determined by measuring molar residue ellipticity at 222 nm in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and a range of urea concentrations from 0 to 3 M. Note that both proteins exhibit a plateau of stability at urea concentrations up to 1 M.

DYSR11-17 and UTROR11-16 (Fig. 3) and found that each exhibited a plateau of stability in urea concentrations as high as 1 M. Such a plateau has been demonstrated as a specific property of stably folded dystrophin rod fragments (29, 32). Furthermore, thermal melting profiles, determined from plots of molar ellipticity at 222 nm over a wide range of temperatures, indicated that each recombinant protein had a T_m of approximately 56 °C, consistent with those previously reported for stable dystrophin fragments (30) and β -spectrin (33). The results of the circular dichroism, gel filtration, and sedimentation velocity analysis collectively indicate that the recombinant rod domain fragments adopted stable, helical conformations, consistent with native folds.

We measured the F-actin binding properties of both proteins by high speed cosedimentation analysis using 5 μ M F-actin and 5 μ M recombinant protein and found that, while DYSR11-17 qualitatively bound F-actin, UTROR11-16 exhibited no actin binding activity (Fig. 4A). In order to quantitatively measure the actin binding activities of the two proteins, we performed F-actin cosedimentation assays using 5 μ M actin and a range of recombinant proteins from 0.5 to 100 μ M. Over this wide range of concentrations, DYSR11-17 bound actin saturably, with an apparent K_d of $7.3 \pm 1.3 \mu$ M and a molar stoichiometry of 1:5 ($B_{max} = 0.19 \pm 0.07$) (Fig. 4B). Over this wide range of concentrations, we again observed no binding activity of UTROR11-16, demonstrating that the middle rod domain of utrophin lacks the actin binding activity of dystrophin. In order to verify that actin binding by DYSR11-17 was due to electrostatic attractions, we performed F-actin cosedimentation using 5 μ M actin and 5 μ M DYSR11-17 in buffer containing a wide range of NaCl concentrations. We found the binding of DYSR11-17 to F-actin to be highly sensitive to increasing ionic strength, exhibiting an IC_{50} of 200 mM, with binding essentially abolished at 400 mM (Fig. 5). These results further support the electrostatic nature of the interaction of the dystrophin rod domain with F-actin.

DISCUSSION

We had previously proposed a mechanism for the interaction of the dystrophin rod domain with F-actin, whereby a cluster of basic spectrin-like repeats near the middle of the rod domain interacts with the actin filament via electrostatic interactions (1). Identification of this binding site was first made through

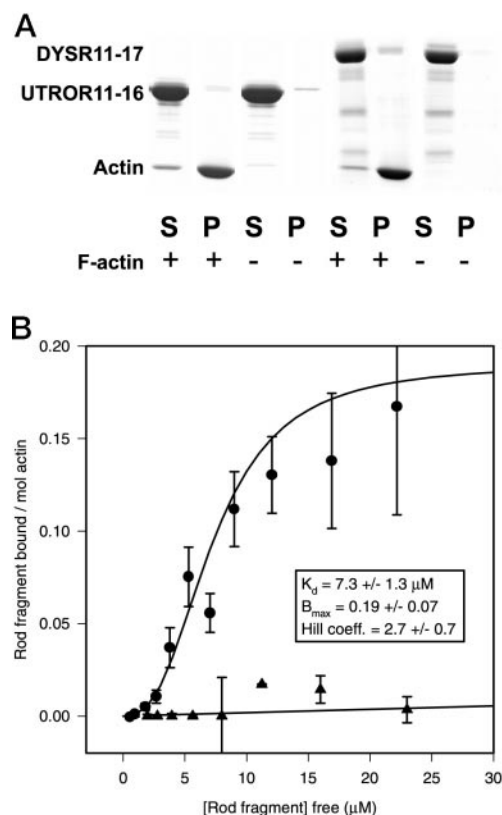


FIG. 4. A, cosedimentation of rod domain fragments with F-actin. Shown are Coomassie Blue-stained SDS-polyacrylamide gels of supernatants (S) and pellets (P) following a 30-min incubation and $100,000 \times g$ centrifugation of 5 μ M purified, recombinant rod domain fragments in the presence (+) or absence (-) of 5 μ M F-actin. B, binding isotherms of rod domain fragments binding to F-actin. DYSR11-17 (circles) or UTROR11-16 at concentrations ranging from 0.5 to 100 μ M was incubated with 5 μ M F-actin and centrifuged at $100,000 \times g$. The amount of protein free or bound to actin was determined densitometrically from Coomassie Blue-stained gels of supernatant and pellet fractions as shown in Fig. 5. Data points shown (\pm S.E.) are the averages of at least two independent experiments. Each individual experiment was curve-fitted by nonlinear regression as described under "Experimental Procedures." The curves shown represent values for B_{max} and K_d determined by averaging the values of the individual experiments.

actin binding analysis of fragments obtained by limited proteolysis of native dystrophin and confirmed with recombinant proteins designed to best represent the proteolytic rod domain fragment that retained actin binding activity (1, 13). The recombinant protein DYS1416, encoding rod domain repeats 11-14, was found to bind F-actin with 14 μ M affinity and 1:1 stoichiometry (1). In contrast, the recombinant protein DYS1030, encoding rod domain repeats 7-10, exhibited no actin binding activity at concentrations as high as 20 μ M. Sequence analysis further indicated that three of the four spectrin-like repeats in DYS1416 were basic, while only one of four repeats in DYS1030 was basic, which led us to hypothesize that DYS1416 bound F-actin largely through electrostatic interactions. In support of this hypothesis, the binding of DYS1416 to F-actin was dramatically inhibited by increasing NaCl concentrations (1). Sequence analysis, however, further indicated that the cluster of basic repeats was not limited to the portion of the rod domain represented by DYS1416 (Fig. 1). Instead, we found that two additional basic repeats were contained within the dystrophin sequence spanning repeats 11-17, suggesting that this region may form a more extensive electrostatic association with the actin filament. In the present study, we have demonstrated that the recombinant protein DYS11-17, encompassing the most contiguous cluster of basic repeats found in dystrophin, bound F-actin with approximately 2-fold higher affinity

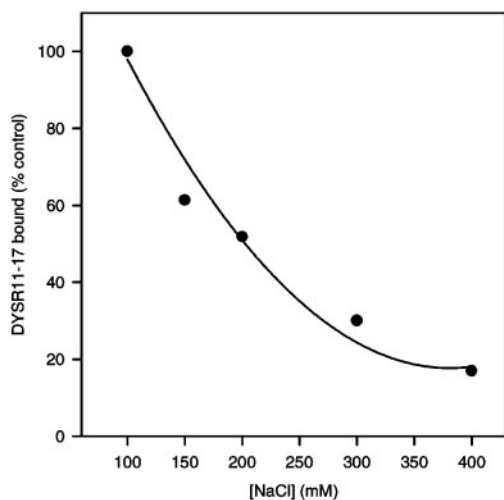


FIG. 5. **NaCl sensitivity of DYSR11-17 binding to F-actin.** 5 μ M DYSR11-17 was incubated with an equimolar concentration of F-actin in actin binding buffer containing a range of NaCl concentrations (100–400 mM) and subjected to cosedimentation analysis as described in Fig. 5. Average binding data ($n = 2$) are expressed as the percentage of binding measured in 100 mM NaCl.

and 5-fold lower stoichiometry (Fig. 4B) than was previously measured for DYS1416 (1). While available data indicate that a single, basic spectrin-like repeat is incapable of binding F-actin (1, 13, 34), our previous (1) and present results indicate that concatenation of three or more basic spectrin-like repeats results in rod domain fragments with actin binding properties that directly correlate with the number of basic repeats present.

We also expressed and characterized a recombinant protein, UTROR11-16, which represented the portion of the utrophin rod domain most similar (by sequence identity) to the basic, actin binding region of the dystrophin rod domain. Although DYS11-17 and UTROR11-16 are 41% identical (64% similar) in primary structure, the predicted pI of DYSR11-17 is 7.44, while that of UTROR11-16 is 4.86 (Fig. 1). Five of seven repeats in DYSR11-17 exhibit pIs over 7.5, while none of the six repeats in UTROR11-16 has a pI greater than 6.3 (Fig. 1). Significantly, the distribution of basic and acidic repeats in dystrophin and utrophin is conserved across all vertebrate dystrophins and utrophins in the data base, including human, mouse, dog, rat, and chicken, suggesting that any charge-specific differences between the two proteins are functionally significant. Most importantly, we have demonstrated that UTROR11-16 lacks the actin binding activity (Fig. 4, A and B) that we have consistently observed in proteolytic fragments and recombinant proteins corresponding to the basic repeat cluster of dystrophin. While we cannot definitively rule out the possibility that UTROR11-16 lacks actin binding activity because it is improperly folded, other results (Table I, Figs. 2–4) suggest that DYSR11-17 and UTROR11-16 exhibit grossly similar physical and structural properties. By gel filtration chromatography and sedimentation velocity analysis, we determined that each recombinant protein was an elongated, highly soluble monomer. Thus, like dystrophin (31, 35, 36), the middle rod domain of utrophin shows no propensity to dimerize. The Stokes radius, sedimentation, and frictional coefficients of DYSR11-17 were slightly greater than those of UTROR11-16, which is wholly consistent with its greater predicted and observed molecular weights and the fact that DYSR11-17 encodes one additional spectrin-like repeat. Furthermore, circular dichroism and UV spectral analysis revealed both proteins to be highly stable, helical structures. Thus, we conclude that the failure of UTROR11-16 to bind F-actin is due to the acidic nature of the repeats comprising this protein, which lends

further support to our hypothesis that it is an excess of basic residues that confers actin binding activity to the repeats present in the dystrophin middle rod domain.

While previous studies have identified functional differences between comparable domains of dystrophin and utrophin (25, 37, 38), ours is the first study to identify a functional difference in the large rod-like domain. Furthermore, this is the first study to provide a structural basis for an observed functional difference between dystrophin and utrophin. We previously demonstrated that the two spatially separated actin binding sites in dystrophin enable it to form an extended, lateral association with F-actin and protect actin filaments from depolymerization *in vitro* (13, 35). More recently, we have obtained preliminary evidence that dystrophin also stabilizes costameric actin filaments *in vivo*.² Because the amino-terminal and basic middle rod domains must be covalently associated to stabilize F-actin (35) and utrophin lacks the middle rod domain actin binding activity of dystrophin (Figs. 5 and 6), we hypothesize that utrophin may lack the filament-stabilizing activity of dystrophin. Such an activity may be particularly important in stabilizing the costameric cytoskeleton associated with the sarcolemmal membrane. Interestingly, the costameric cytoskeleton is disrupted in dystrophin-deficient muscle although utrophin is up-regulated and retained in a costameric pattern (39).

Finally, our results have implications for the potential of utrophin up-regulation to fully replace the function(s) normally served by dystrophin, which is under active investigation as a therapy for dystrophin-deficient muscular dystrophies. Studies in transgenic *mdx* mice clearly indicate that expression of a dystrophin molecule with only one actin binding site (either N-terminal or middle rod domain) or utrophin can significantly compensate for the absence of full-length dystrophin (12, 40–42). However, other data call into question whether utrophin overexpression will extrapolate to similar functional recoveries in human patients. Unlike the *mdx* mouse (18, 19), there is no apparent correlation between increased utrophin expression and decreased muscle cell necrosis in patients with Duchenne or Becker muscular dystrophies (43–45). Likewise, transgenic *mdx* mice expressing a dystrophin deleted for only the actin binding middle rod domain exhibit a very mild phenotype (40, 41). In contrast, human muscular dystrophy patients with middle rod domain deletions can present with phenotypes that range from mild to severe (46–48). Most notably, one frequently cited study described a patient with a middle rod domain deletion who “was able to walk with the aid of a stick at age 61” (46), while a later study (48) from the same group identified additional patients with similar deletions who became wheelchair bound by age 16. It has been suggested that the sarcolemmal membrane of mouse skeletal muscle may experience less mechanical stress per unit area due both to the smaller size of the organism and to the smaller caliber of individual skeletal muscle fibers compared with humans (19, 49). Thus, we think it possible that two actin binding sites may confer unique mechanical properties to the dystrophin/F-actin interaction that are more important to muscle membrane stabilization in larger animals. Consistent with this idea, utrophin up-regulation also does not appear to ameliorate the severe dystrophic phenotype of dystrophin-deficient dogs (50). While we are hopeful that utrophin overexpression will prove efficacious as a treatment for Duchenne muscular dystrophy, any beneficial effect of utrophin on the function of human dystrophic muscle remains to be demonstrated. Until then, the present data suggest that the basic middle rod domain is a unique and important functional domain of dystrophin that warrants further investigation.

² I. Rybakova and J. Ervasti, unpublished results.

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