Most studies aimed at characterizing the utrophin-actin interaction have focused on the amino-terminal tandem calponin homology domain. However, we recently reported evidence suggesting that spectrin-like repeats of utrophin also participate in binding to actin. Here we expressed several recombinant fragments encoding the utrophin amino-terminal domain alone or in combination with various numbers of spectrin-like repeats. We further quantitatively characterized the actin binding properties of each recombinant utrophin fragment using a high-speed sedimentation assay. To evaluate the capacity of each protein to stabilize actin filaments, we compared the effect of recombinant utrophin fragments and full-length utrophin on 6-propionyl-2-(N,N-dimethylamino)naphthalene. Our results suggest that, whereas the amino-terminal domain and first 10 consecutive spectrin-like repeats recapitulate the actin binding activity of full-length utrophin more faithfully than the amino-terminal domain alone. These findings support the model for lateral association of utrophin along the actin filament and provide the molecular basis for designing the most effective utrophin “mini-genes” for treatment of dystrophinopathies.

Utrophin is a large cytoskeletal protein that is abundantly expressed throughout fetal and regenerating muscles but is down-regulated at birth and restricted to the myotendinous and neuromuscular junctions of normal adult muscle (1). It belongs to the spectrin superfamily of proteins, all containing an actin binding region composed of single or multiple copies of tandem calponin homology (CH)1 domains. Utrophin also contains a large rod-shaped domain consisting of 22 spectrin-like repeats and cysteine-rich/carboxyl-terminal domains important for utrophin localization to the sarcolemma. Utrophin is highly homologous to dystrophin, the protein absent or defective in Duchenne and Becker muscular dystrophies (2, 3). Both utrophin and dystrophin bind the same complement of proteins and link actin cytoskeleton to the extracellular matrix (4–7). This link is believed to be important to mechanically stabilize the sarcolemmal membrane from shear stresses imposed during eccentric muscle contraction (8–10). In the absence of dystrophin, the link between actin cytoskeleton and extracellular matrix is disrupted, and the compromised sarcolemmal integrity leads to muscular dystrophy (5, 10). Although the precise role of utrophin is less clear, it may have a similar function to dystrophin in membrane stabilization, as the overexpression of full-length utrophin transgene in the dystrophin-deficient mdx mouse resulted in a recovery for all known parameters of dystrophic phenotype (11). Notably, utrophin overexpression rescued the mechanical linkage between costameric actin and the sarcolemma of mdx mouse muscle (12).

Most studies aimed at characterizing the actin binding function of utrophin have focused on its amino-terminal tandem CH domain encoded within the first 261 amino acids (Utr261). Utr261 cosedimented with F-actin in a saturable manner but with low affinity and localized to stress fibers when microinjected into fibroblasts (13, 14). The crystallographic study of Utr261 revealed that its two CH domains are separated by an extended α-helix, suggesting that this actin binding region of utrophin may be more flexible than was concluded previously from the crystal structure of the analogous domain of fimbrin (15, 16). Electron microscopy and image reconstruction analysis of Utr261 complexed with actin filaments established that the flexibility of CH domains within the utrophin amino terminus results in two different modes or stoichiometries of utrophin-actin interaction (17). When existing crystal structures of actin binding domains of dystrophin, plectin, fimbrin, and α-actinin are considered, these results suggest that the spectrin superfamily of proteins creates diverse structures with actin filaments because of the ability of the CH modules to rearrange on multiple binding sites within the actin subunit (18–21). Although characterization of Utr261-actin interaction is approaching atomic resolution, biochemical studies of truncated and full-length utrophin isoforms suggest an important role for spectrin-like repeats of the rod domain in the utrophin-actin interaction. Briefly, full-length utrophin has been shown to bind actin with substantially higher affinity and through more extensive lateral association when compared with Utr261 (12). A truncated utrophin isoform expressed in C6 glioma cells corresponding to the amino-terminal domain and 2.5 spectrin-like repeats had actin binding characteristics intermediate to those measured for full-length utrophin and Utr261 (22).

To better define the molecular epitopes of utrophin-actin interaction and expand our understanding of the diverse actin binding mechanisms employed by spectrin superfamily of proteins, it is therefore necessary to determine how the utrophin rod domain participates in actin binding. In this study, we generated recombinant utrophin fragments encoding the amino-terminal domain and/or different numbers of spectrin-like...
repeats. By gel filtration chromatography and sedimentation velocity analysis, we found that each utrophin protein existed as a highly soluble monomer. The actin binding properties of utrophin proteins were characterized by high-speed sedimentation and PRODAN-labeled F-actin depolymerization assays. Our data indicate that, whereas the 10 spectrin-like repeats of utrophin exhibited no measurable actin binding activity when expressed in the absence of the amino-terminal domain, their presence dramatically enhanced both affinity and capacity of the amino-terminal domain for F-actin and more fully recapitulates the activities measured for full-length utrophin.

EXPERIMENTAL PROCEDURES

Utrophin Proteins—Full-length mouse utrophin was expressed in the baculovirus expression system as described previously (12). DNA fragments encoding the amino terminus and different numbers of spectrin-like repeats of utrophin were PCR-amplified from the Bluescript KS II+ vector containing the full-length mouse utrophin sequence (23). The sequence-verified PCR products were ligated into pFastBac1 transfer plasmid (Invitrogen) for the site-specific transposition of an expression cassette into the bacmid. Baculovirus strains prepared from the recombinant bacmids were used to infect SF9 cell monolayers for utrophin fragment expression. Recombinant utrophin fragments were tagged with the FLAG epitope and, therefore, isolated from infected insect cell lysates on Anti-FLAG M2-agarose (Sigma) as described previously for the purification of full-length FLAG-tagged utrophin (12).

Hydrodynamic Analysis—Measurements of the sedimentation coefficient and Stokes radius as well as the calculation of the native molecular weight and frictional coefficient of utrophin proteins were performed as described previously (24, 25).

Actin Binding Analysis—The actin binding properties of utrophin recombinant fragments were measured using the high-speed cosedimentation assay described previously (7). Briefly, increasing amounts of purified utrophin proteins incubated with 6 μM muscle F-actin (cytoskeleton) were centrifuged at 100,000 × g for 20 min. The amount of free and bound protein was determined densitometrically from Coomassie Blue-stained gels of resulting supernatants and F-actin pellet fractions. Binding data were fitted to the Michaelis-Menten equation by non-linear regression analysis (26).

PRODAN Actin Depolymerization Assay—PRODAN-labeled actin (27) was the kind gift of Dr. G. Marriott (University of Wisconsin, Madison). 2 μM PRODAN-labeled G-actin was polymerized alone or in the presence of utrophin proteins in a buffer containing 10 mM Tris-HCl, pH 8.0, 0.1 mM ATP, 0.1 mM NaCl, 2 mM MgCl₂, and 0.2 mM dithiothreitol. Total reaction volume was 60 μl. Assembled actin filaments were induced to depolymerize by dilution to 0.1 μM in low salt buffer conditions (0.005 mM NaCl and 0.01 mM MgCl₂). Total reaction volume after the dilution was 1.2 ml. Fluorescence measurements were performed at 25 °C in an SLM-AMINCO AB2 fluorescence spectrophotometer (ThermoElectron) at an excitation wavelength of 385 ± 4 nm and an emission wavelength of 465 ± 4 nm.

RESULTS

To explore what portion of the utrophin rod domain is directly involved in binding actin filaments, we produced four recombinant proteins encoding the amino-terminal domain plus 3, 6, 9, and 10 spectrin-like repeats of utrophin (Fig. 1A). Increasing numbers of repeats were added to the N-terminal actin binding domain of utrophin based on the alignment of triple-helical repeats in α- and β-spectrin, dystrophin, and utrophin (6). We also generated a construct encoding the amino-terminal domain alone and a construct corresponding exclusively to the first 10 spectrin-like repeats of utrophin. All of the recombinant utrophin fragments were tagged with the FLAG epitope and purified using Anti-FLAG M2-agarose (Fig. 1B).

We first characterized the hydrodynamic properties of each purified utrophin fragment. 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 consistent with its predicted monomeric molecular weight, therefore indicating that each protein existed predominantly as a highly soluble monomer in solution. As estimated by the frictional coefficient, utrophin fragments assumed an elongated shape in the solution and became increasingly elongated as more spectrin-like repeats were added (Table I). This observation is consistent with the available molecular dimensions (16, 28) and electron microscopy images of utrophin (12).

We further characterized the actin binding properties of each utrophin fragment by high-speed cosedimentation analysis using 6 μM F-actin and increasing concentrations of the utrophin proteins (Fig. 2A). To determine the exact number of spectrin-like repeats necessary for a 0.15 μM affinity and 1:14 stoichiometry as measured for the full-length utrophin-actin interaction (12), all experiments were performed in parallel with full-length utrophin. Fitting the binding data obtained from three independent experiments to the Michaelis-Menten equation showed that the fragment (UtrN-R10, encodes UtrNT plus spectra repeats 1–10, see Fig. 1A) corresponding to the amino-terminal actin binding domain and first 10 spectrin-like repeats bound F-actin with parameters highly similar to those measured for full-length utrophin (Figs. 2, B, and C, and 3). The isolated amino-terminal domain (UtrNT) exhibited the lowest affinity with a K₅₀ of 18.6 ± 5.31 μM, which is consistent with the dissociation constants measured for actin binding of the amino-terminal domain of utrophin expressed in Escherichia coli (12.9 ± 2.1 μM (14), 19.2 ± 2.2 μM (13)). Furthermore, UtrN-R3 and truncated C6 glioma cell utrophin composing the amino-terminal domain and the first 25 spectrin-like repeats bound actin with similar K₅₀ values of 2.13 (Fig. 3A) and 2.2 ± 2 μM (22), respectively. Both UtrN-R6 and UtrN-R9 bound F-actin with affinities intermediate to those observed for UtrN-R3 and Utr. Thus, UtrNT, UtrN-R3, UtrN-R6, UtrN-R9, and UtrN-R10 each bound actin filaments with progressively higher affinity.
shown in Fig. 4 alone or UtrNT mixed with UtrR1–R10 at 1:1 molar ratio. As measurable effect on either the fraction when UtrNT was present. Next, UtrR1–R10 had no affinity and stoichiometry of the interaction. the following 10 spectrin-like repeats dramatically enhance the to have the most dramatic effect on actin binding, whereas inclusion of additional repeats up to 10 effected linear increases in affinity and stoichiometry. The measured affinity and stoichiometry of UtrN-R10 were not different from full-length utrophin (Fig. 3).

Interestingly, the construct encoding 10 spectrin-like repeats without the amino-terminal domain (UtrR1–R10) exhibited no actin binding activity over the range of concentrations from 0.5 to 60 μM (not shown). To test whether UtrR1–R10 can bind UtrNT and possibly alter the actin binding properties of UtrNT in trans, we cosedimented F-actin in the presence of UtrNT alone or UtrNT mixed with UtrR1–R10 at 1:1 molar ratio. As shown in Fig. 4A, no UtrR1–R10 was detected in the pellet fraction when UtrNT was present. Next, UtrR1–R10 had no measurable effect on either the K_d or B_max versus spectrin repeat number (Fig. 3). As expected, the homologous but acidic utrophin domain, has been shown to bind actin independently from its adjacent amino-terminal calponin homology domain (30). In contrast, a cluster of basic repeats, 11–17 of the dystrophin rod domain, has been shown to bind actin independently from its amino terminus (7, 31). The homologous but acidic utrophin middle rod repeats 11–16 failed to bind actin (25), which, together with the actin binding characteristics of full-length utrophin, led us to propose a model wherein the amino-terminal domain and first 10 spectrin-like repeats act in concert to position utrophin laterally along the actin filament (12). Here, the recombinant utrophin fragment UtrN-R10 bound F-actin with essentially the same properties as full-length utrophin (Figs. 2 and 3). Interestingly, UtrR1–R10, lacking the amino-terminal domain, exhibited no actin binding activity at concentrations as high as 60 μM. The interaction of spectrin-like repeats may be too weak for measurement by high-speed sedimentation assay. Alternatively, expression of the amino-terminal tandem CH domain in cis with spectrin repeats may induce conformational changes that create or expose new actin binding sites on utrophin or new utrophin binding regions on actin. Considering that the amino-terminal domain and spectrin-like repeats of utrophin act as an actin binding unit and that the affinity and capacity of utrophin fragments for actin increases sequentially with the added number of spectrin-like repeats (Fig. 3), we suggest the following mechanism for utrophin-actin interaction. (i) The amino-terminal domain is necessary for primary interaction between utrophin and actin. (ii) The interaction of the amino-terminal domain and actin results in the conformational changes that allow for additional contacts between the spectrin-like repeats and actin filaments. (iii) The spectrin-like repeats thus stabilize actin filaments and increase the affinity and lateral association of the binding.

More detailed ultrastructural analysis is necessary to determine the precise contribution of repeats to utrophin association with actin. To date, the only reconstructions of this kind were performed for actin filaments decorated with the amino-termi-

### Table I

| Protein      | UtrNT | UtrN-R3 | UtrN-R6 | UtrN-R9 | UtrN-R10 | UtrR1–R10 | Utr |
|--------------|-------|---------|---------|---------|----------|-----------|-----|
| Predicted M_r | 30,654 | 74,233  | 117,394 | 154,226 | 165,497  | 130,681   | 392,515 |
| Denatured M_r| 33,253 | 79,568  | 117,589 | 152,489 | 168,009  | 129,520   | 379,000 |
| Native M_r   | 31,750 | 82,054  | 119,943 | 156,922 | 172,790  | 131,847   | 404,000 |
| Stokes radius, nm | 2.7  | 4.2    | 5.4    | 6.7    | 6.7     | 5.9       | 9.1  |
| Sedimentation coefficient, s | 2.7  | 4.6    | 5.1    | 6.0    | 6.0     | 5.2       | 10.4 |
| Frictional coefficient | 1.22 | 1.44   | 1.67   | 1.87   | 1.81    | 1.75      | 1.86 |

DISCUSSION

Traditionally, the spectrin-like repeats of utrophin have been viewed as spacer modules that serve to separate important functional domains at the amino and carboxyl termini. More recently, spectrin repeats in several different proteins have been shown to interact with a diverse distinct array of proteins, including actin (29). Spectrin repeats are necessary for the full actin binding activity of both dystrophin and spectrin. In β-spectrin, the first repeat exhibited no detectable actin binding activity by itself, but it increased the affinity of the adjacent amino-terminal calponin homology domain (30). The interaction of spectrin-like repeats may be too weak for measurement by high-speed sedimentation assay. Alternatively, expression of the amino-terminal tandem CH domain in cis with spectrin repeats may induce conformational changes that create or expose new actin binding sites on utrophin or new utrophin binding regions on actin. Considering that the amino-terminal domain and spectrin-like repeats of utrophin act as an actin binding unit and that the affinity and capacity of utrophin fragments for actin increases sequentially with the added number of spectrin-like repeats (Fig. 3), we suggest the following mechanism for utrophin-actin interaction. (i) The amino-terminal domain is necessary for primary interaction between utrophin and actin. (ii) The interaction of the amino-terminal domain and actin results in the conformational changes that allow for additional contacts between the spectrin-like repeats and actin filaments. (iii) The spectrin-like repeats thus stabilize actin filaments and increase the affinity and lateral association of the binding.
Although these reconstructions were generated from unsorted actin filaments and therefore may not depict potential heterogeneity in association (33), they nonetheless revealed densities attributable to both the tandem CH domain and the spectrin repeat in close apposition with actin (32). Moreover, the spectrin repeat density was positioned in a location and orientation similar to those adopted by tropomyosin when bound to F-actin.

As is clearly evident from Figs. 2 and 3, the addition of spectrin-like repeats 1–3 subsequent to the amino-terminal domain of utrophin had a more dramatic effect on the $K_d$ and $B_{\text{max}}$ of actin binding than did the consecutive inclusion of repeats 4–10. The greater contribution of spectrin repeats 1–3 to the actin binding properties of utrophin may be because of unique sequence/structural features or their closer proximity to the amino-terminal tandem CH domain. To address these issues, future analyses of constructs encoding the amino-terminal domain expressed in cis with proximal (repeats 1–3) versus distal (repeats 4–6 or 7–9) spectrin repeats will be informative. It may also be interesting to assess whether spectrin repeats from other proteins can also enhance actin binding of the utrophin amino-terminal tandem CH domain. However, a chimeric dystrophin transgene encoding the amino- and carboxyl-terminal domains of dystrophin plus the four-repeat rod domain of $\alpha$-actinin 2 was incapable of correcting the phenotypes of the dystrophin-deficient $mdx$ mouse even though it rescued assembly of the dystrophin-glycoprotein complex at the sarcolemma (34).

From biochemical and structural studies, highly related proteins appear to have very diverse mechanisms of actin binding driven by rearrangement of the tandem CH domains. Reconstruction of the fimbrin tandem CH domain docked on actin filaments revealed that it bound actin in compact globular form (15). In contrast, the amino-terminal tandem CH domain of
both dystrophin and utrophin bound actin in extended conformation with the two CH modules widely separated (33). In plectin, the two CH domains were in closed conformation in the crystal, whereas binding to actin filaments induced an open conformation (19). Our results suggest that even greater diversity may arise when actin binding of tandem CH domains is modulated by spectrin-like repeats or other repeat domains such as the antiparallel β-sheet modules in filamin (36, 37).

With regard to functional diversity, it will be of interest to evaluate actin binding characteristics of dystrophin fragments comprising its amino terminus and 10 consecutive spectrin-like repeats and to directly compare molecular epitopes involved in dystrophin and utrophin interaction with actin. More generally, our results raise the possibility that the combined presence of a CH-type actin binding domain and spectrin-like repeats may enable some members of the plakin family of cytolinkers to laterally bind and stabilize actin filaments (38). In support of this possibility, actin filaments associated with

![Figure 4](image1.png)  
**FIG. 4.** Spectrin-like repeats do not alter the actin binding properties of the utrophin tandem CH domain when presented in trans. A, Coomassie Blue-stained SDS-polyacrylamide gel of 100,000 g supernatants (S) and pellets (P) of 6 μM F-actin incubated with UtrR1–R10 (S1, P1), UtrNT (S2, P2), or an equimolar mixture of UtrR1–R10 and UtrNT (S3, P3). Both UtrNT and UtrR1–R10 were present at 5 μM. B, increasing amounts of UtrNT alone (C) or in the presence of equimolar concentrations of UtrR1–R10 (C) were cosedimented with F-actin at 100,000 g. The amount of free and bound UtrNT was determined densitometrically from Coomassie Blue-stained gels of supernatant and pellet fractions as shown in A. Nonlinear regression analysis yielded apparent Kd values of 27.8 ± 11.6 and 23.6 ± 13.7 for UtrNT and UtrNT plus UtrR1 binding to F-actin, respectively. C, Coomassie Blue-stained SDS-PAGE and A280 profile of fractions eluted from a Sephacryl-200 column loaded with an equimolar mixture of UtrNT and UtrR1–R10.

![Figure 5](image2.png)  
**FIG. 5.** Effect of utrophin constructs on forced depolymerization of actin filaments. 1 μM PRODAN-labeled F-actin was preincubated alone (Actin) or in the presence of utrophin constructs. Depolymerization was monitored by measuring fluorescence decline after dilution of F-actin to 0.1 μM into low salt buffer conditions. A–C, graphs representing the results of typical experiments where the utrophin:actin molar ratio was 1:1, 1:12, or 1:3, respectively.
Actin Binding Site of Utrophin

microtubule-bound microtubule actin cross-linking factor were recently found to be more resistant to depolymerization induced by latrunculin B (39).

Finally, our results also have clinical implication, as they provide a molecular basis for the greater effectiveness of full-length utrophin in rescuing the phenotypes of dystrophin-deficient muscle compared with a utrophin mini-gene deleted for spectrin-like repeats 4–19 (11, 35, 40). Our data may further lead to the design of shorter but still fully functional utrophin mini-genes useful in the treatment of Duchenne muscular dystrophy.

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