In vitro expressed dystrophin fragments do not associate with each other

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Abstract Dystrophin, a component of the muscle membrane cytoskeleton, is the protein altered in Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD). Dystrophin shares significant homology with other cytoskeletal proteins, such as α-actinin and spectrin. On the basis of its sequence similarity with α-actinin and spectrin, dystrophin has been proposed to function as dimer. However, the existence of both dimers and monomers have been observed by electron microscopy. To address this apparent discrepancy, we expressed dystrophin fragments composed of different domains in an in vitro translation system. The expressed fragments were tested for their ability to interact with each other and full-length dystrophin by both immunoprecipitation and blot overlay assays. These assays were successfully used to demonstrate the dimerization of α-actinin and spectrin, yet failed to detect any interaction between dystrophin fragments. Although these in vitro results do not prove that dystrophin is not a dimer in vivo, they do indicate that this interaction is not like that of the α-actinin and spectrin.

Key words: Dystrophin; In vitro translation; Dimerization; Immunoprecipitation; Blot overlay

1. Introduction

Dystrophin is a large cytoskeletal protein found at the muscle sarcolemma and membrane specializations of some neurons [1,2]. Mutations of the gene encoding dystrophin have been shown to be causative for X-linked Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD) [3,4]. The 3685-amino-acid sequence shares homology with a large family of actin-binding cytoskeletal proteins that includes α-actinin and spectrin [5]. The functions of dystrophin are still emerging but, based on its subcellular localization and homology with spectrin, it is thought to maintain the integrity of the muscle membrane and to protect myofibers from damage during contraction and relaxation [6].

Dystrophin contains four large structurally distinct domains [7]: an N-terminal actin-binding domain, an internal rod domain that contains 24 repeat units structurally homologous to those found in spectrin, a cysteine-rich region which has homology to EF-hand calcium-binding domain found in calmodulin, α-actinin and β-spectrin, and, lastly, a unique carboxyl terminus that only shows sequence similarity to an autosomal 395 kDa dystrophin-related protein (DRP), utrophin [8], and the human homologue of the Torpedo 87 kDa post-synaptic membrane protein, dystrobrevin [9]. Recently, a motif that contains two highly conserved tryptophan residues known as WW domain has also been identified at the end of the rod domain [10].

Since both spectrins and α-actinin exist as anti-parallel dimers [11,12], given its overall structural homology with them, dystrophin is also expected to function as a dimer. However, electron microscopy analyses of rotary-shadowed images of dystrophin have reported that the molecular shape of dystrophin to be either a 175 nm flexible rod monomer [13,14] or a 120 nm dumbbell-shaped dimer [15]. At this moment, there is no clear biochemical data to support or reject either finding. Meanwhile, in vivo analysis of dystrophin has been hindered by factors, such as its enormous size (427 kDa), low abundance in muscle (0.002%) and tight association with other dystrophin-associated proteins.

Because of this difficulty, we examined the potential dimerization of dystrophin by expressing different fragments of dystrophin in vitro and tested their ability to interact with each other in both immunoprecipitation and blot overlay assay. Muscle homogenates containing full-length dystrophin were also tested for their ability to interact with the in vitro translated dystrophin fragments in the overlay assay. In neither system could we detect association of any dystrophin fragment with itself or other fragments. However, under the same conditions, we clearly documented the dimerization of α-actinins and spectrins. Our results suggest that the biochemical properties of dystrophin are different from α-actinin and spectrin in spite of their significant structural homology. In addition, our findings support the electron microscopic studies that dystrophin is a monomer.

2. Materials and methods

2.1. Subcloning of dystrophin fragments into expression vectors

PCR primers were designed to amplify regions of cDNA corresponding to the different domains of dystrophin from the full-length dystrophin cDNA clone, BTFL [4]. To maintain the accuracy of the amplification, Expand™ High Fidelity DNA polymerase which has a 3′–5′ exonuclease proofreading activity was used (Boehringer Mannheim, Germany). The 5′ and 3′ ends of each clone were sequenced by the dideoxy-termination method using *Taq* polymerase to confirm the accuracy of the cloning procedures. The constructs, along with their expressed dystrophin sequences and the PCR primers are listed in Table 1.

2.2. In vitro translation of dystrophin sequences

The expression vectors used in this study, pMGT-1 and pFHR-1, have been previously described in detail [16]. The pFHR-1 vector allowed the translation of the protein of interest with a N-terminal fusion polypeptide (MDYKDDDK) called flag (Kodak, Rochester, NY). Briefly, each construct was radiolabeled with either 0.2 μCi of L-[U-^{14}C]leucine (>300 mCi/mmol; Amersham Corp., Arlington Heights, IL) or 40 μCi L-[^{35}S]methionine (>1000 Ci/mmol) and translated in vitro by the TNT T7 coupled reticulocyte lysate system according to the manufacturer's protocol (Promega Corp., Madison, WI).

2.3. Immunoprecipitation experiments

Five microliters of each of the two different in vitro translated dystrophin fragments to be tested were incubated on ice for 2 h with 40 μl of TBST buffer (10 mM Tris-HCl (pH 8.0), 0.1% Tween 20, 150 mM NaCl). Antibody directed against one of the two dystro-

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phin fragments (1.5–10 μg) was then added to the mixture and incubated on ice for another hour. The complex was precipitated by adding 50 μl of 50% protein G–Sepharose (Sigma Chem. Co., St. Louis, MO) and incubating on ice for another 30 min. The mixture was then pelleted at 10000×*g* for 2 min at room temperature, washed 3 times with 1 ml of TBST buffer and analyzed on 8.5% SDS-PAGE gel as previously described [16]. All gels were exposed to a storage phosphor plate after being fixed and dried. The plate was scanned by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed by the ImageQuant software (Molecular Dynamics).

The immunoprecipitation experiments were also carried out under different salt concentrations (150–500 mM), detergent compositions, (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and pH (7.4 and 8.0). They all produced results similar to those obtained with TBST buffer.

2.4. Trypsin digestion of in vitro translated dystrophin fragments

In vitro translated [^{35}S]methionine-labeled dystrophin fragments were immunoprecipitated by the appropriate antibody as described above. The immunoprecipitated dystrophin fragment was then incubated with trypsin in an enzyme to protein ratio of approximately 1:50 in incubation buffer (100 mM Tris-HCl (pH 8.0), 125 mM sucrose, 2.5 mM HEPES) at 37°C for different time intervals and was analyzed on 12% SDS-PAGE gel.

2.5. Blot overlay experiments

In vitro translated dystrophin fragments (or human skeletal muscle homogenates) were separated by 8.5% or 10% SDS-PAGE gel and transferred overnight onto nitrocellulose membrane (constant current of 70 mA at room temperature). The human skeletal muscle homogenates were isolated according to method described previously [17]. The membrane was blocked at 4°C for 2 h in blocking buffer (0.1% gelatin, 5% bovine serum albumin, 0.1% Tween 20 in 1×PBS, pH 7.5). It was then incubated with the in vitro translated [^{35}S]methionine-labeled dystrophin fragment in overlay buffer (150 mM NaCl, 20 mM HEPES, 2 mM $MgCl_2$, 5% bovine serum albumin, pH 7.5) at a concentration of 10 μl of translation mix per milliliter of overlay buffer [18]. The incubation was carried out overnight at 4°C. After five washes with 20 ml of overlay buffer, the blot was dried briefly and exposed to a storage phosphor plate and analyzed by the PhosphorImager as described above.

3. Results

3.1. Expression of dystrophin fragments in vitro

The in vitro expression of dystrophin has been shown to be limited to 150 kDa [16]. Expression of larger size products often results in multiple smaller fragments, presumably due to degradation and inefficient translation. To circumvent this problem, it became necessary to break down dystrophin into smaller sizes. Eight overlapping constructs were designed to encompass the entire dystrophin sequence (Table 1). Each clone encoded a domain of dystrophin as predicted by its secondary structure [7]. The dystrophin fragments were designed to be different sizes so that they could be separated and distinguished by SDS-PAGE gels.

Upon in vitro translation, each dystrophin sub-fragment expressed a polypeptide of predicted size with no significant degradation (< 5%) as judged by SDS-PAGE gel and PhosphorImager (Fig. 1). In both Western blots and immunoprecipitation assay, each dystrophin fragment was only recognized by the antibodies specific to the domain it encoded (data not shown). In addition, most fragments produced a pattern of proteolytic cleavage similar to native dystrophin after trypsin digestion (Fig. 2) [19]. All these results suggested that the in vitro translated dystrophin fragments were stable and adopted a conformation similar to the native full-length dystrophin.

Table 1
Construction of expression vectors

[*]Construct	[#]PCR Primers	Cloning Sites	[$]Domain	[@]Expressed Dystrophin Sequence	No.Of Residues	Predicted Size
N828	cccgGtaccATGCTTTGGTGGGAAGAAGTAGAG GATcTcGagtcaATACTCCAGCCAGTTAAG	KpnI/XhoI	N-terminus + repeat 1-4	MGT(dys1-828)	828	95.7 kD
H1-H2	GAAGgtaccATGTTGCCAAGGCCACCT TTactAgTCCACAGTAATCTGCCT	KpnI/SpeI	hinge 1-2	MGT(dys253-717)	465	53.6 kD
H2-R11	ATTTCcatGGCTGTCACCACCACTCAG TTTCTagAACTGTTGCTTTCTTTCTGT	NcoI/XbaI	hinge 2-repeat 11	M(dys668-1566)	899	104.2 kD
R12-H3	CAACccaTGGAGAAATGCTTGAAATTG AGCAGGTACCTaCAACATCAAGGAAGA	NcoI/KpnI	repeat 12-hinge 3	M(dys1567-2467)	901	104.8 kD
R7-R15	AAATccATGGCTGAAGTTGATGTTTTT AGTGTGAATTcGTcaAAAGTTGAGTCT	NcoI/EcoRI	repeat 7-repeat 15	dys1064-1971	908	105.6 kD
R19-H4	GAAAccatgGCTCAAATAAAAGACCTT GTCTctaGAGTTTCATGGCAGTCCT	NcoI/XbaI	repeat 19-hinge 4	M(dys2318-3112)	795	92.4 kD
C2321	ATTGccatggAAATAAAAGACCTTGGGCAG ATGTAGaaTTCTTATAACTTTTTTGT	NcoI/EcoRI	repeat 19-C-terminus	M(dys2319-3685)	1367	157.5 kD
C2979	acttccacCAtGGCACTGCGAGGAGAAATT GTGGAgtcGACTTCCTACATTGTGTCCTCTCTCATTGGCT	NcoI/BamHI	repeat 24-C-terminus	M(dys2980-3685)	706	80.4 kD

[a]Each construct also has a variant version with a N-terminal flag fusion polypeptide (MDYKDDDKGS).
[b]Primers are written in the conventional 5′–3′ direction, the upper one being the forward primer, the lower one being the reverse primer. Nucleotides in capital letter represent the wild-type dystrophin sequence. Nucleotides in small letter indicate a change from the wild-type sequence in order to create a restriction site compatible with the vectors. The created restriction sites were underlined.
[c]Domains of dystrophin were predicted from secondary structure according to Koenig [7].
[d]Single letter amino acid symbols are used. Amino acid numbers for human dystrophin was from Koenig [7].

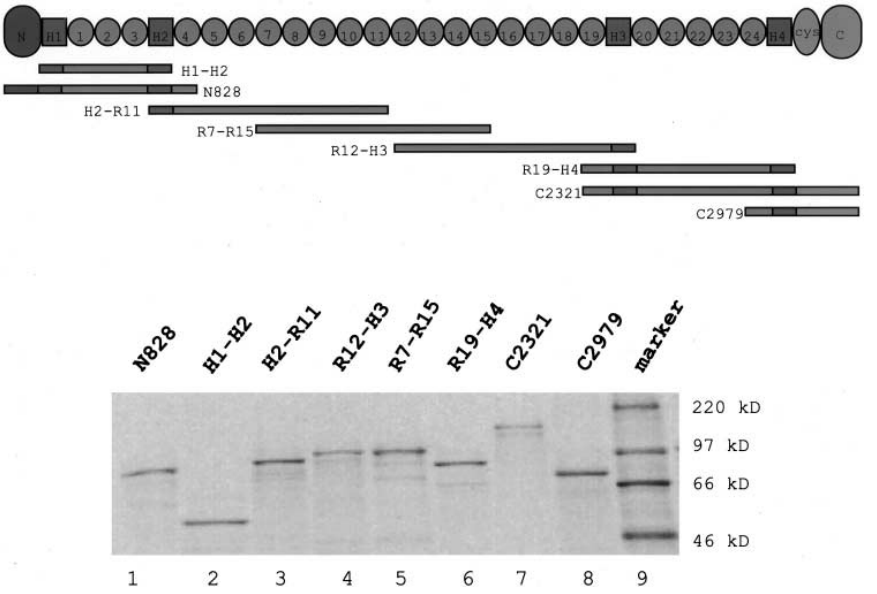

Fig. 1. In vitro translation of dystrophin fragments. Diagram depicts secondary structure of dystrophin. N, N-terminal actin-binding domain; Cys, cysteine-rich domain; C, unique C-terminal domain. The rod domain is represented by squares (hinge region, H) and circles (repeat unit 1–24). The solid bars denote the in vitro translated dystrophin fragments. All reactions were labeled with L-[U-^{14}C]leucine according to Section 2. Lane 1: N828; lane 2: H1–H2; lane 3: H2–R11; lane 4: R12–H3; lane 5: R7–R15; lane 6: R19–H4; lane 7: C2321; lane 8: C2979; lane 9: ^{14}C-methylated high molecular mass standards (Amersham Corp.).

3.2. *Dystrophin fragments did not interact with each other in co-immunoprecipitation assay*

Before we began the systematic screening of dystrophin fragments for specific interactions, we first tested the validity of using the in vitro translated dystrophin fragments by demonstrating that in vitro translated spectrin fragments can associate with each other in co-immunoprecipitation assay providing that they contain the dimerization domains. Clones β_{1-4} encodes the N-terminal domain and the first three repeat units of β-spectrin (100 kDa) and clone α_{19-22} encodes the last three repeat units and the C-terminal domain of α-spectrin (57 kDa). Together, they mediate the interaction between β- and α-spectrin [20,21]. As shown in Fig. 3A, an antibody specific to β-spectrin was able to precipitate both β_{1-4} and α_{19-22} (Fig. 3A, lane 5). In contrast, when assayed with the same antibody, β_{1-4} failed to co-precipitate a α_{19-22} mutant that has two small in-frame deletions which abolished the interchain binding activity (Fig. 3A, lane 7). All three spectrin clones were in vitro translated into a single product of expected size (Fig. 3A, lanes 1–3). No protein was precipitated in the absence of antibody (Fig. 3A, lanes 4 and 6), thus documenting the specific in vitro co-immunoprecipitation of the spectrin chains. β_1-Syntrophin, a dystrophin associated protein, was also demonstrated by the same assay to interact with the C-terminal dystrophin fragment (data not shown). This result was consistent with findings by our group [16] and others using different methods [22].

Given the fact that the end termini of spectrin plays an important role in its dimerization, the terminal domains of dystrophin were tested first. Clone N828 encodes the N-terminal actin binding sites and the first four tandemly repeating units of dystrophin. Clone C2321 encodes the last six repeat

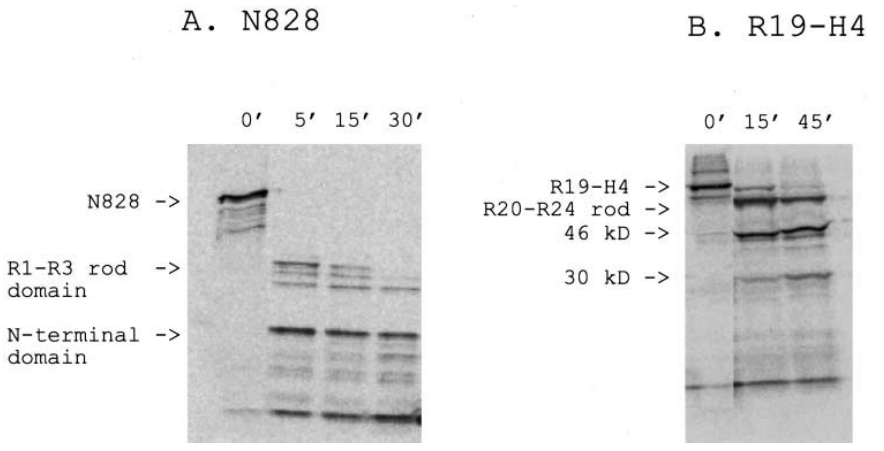

Fig. 2. Trypsin digestion of in vitro translated dystrophin fragments. In vitro translated ^{35}S-labeled dystrophin fragments were digested with trypsin for different intervals of time (0–45 min) according to Section 2. A: N828. B: R19–H4. In both examples, correct folding of the fragment was characterized by the presence of proteolytic resistant subfragments of lower molecular mass that were similar to the trypsin digest of native full-length dystrophin [19].

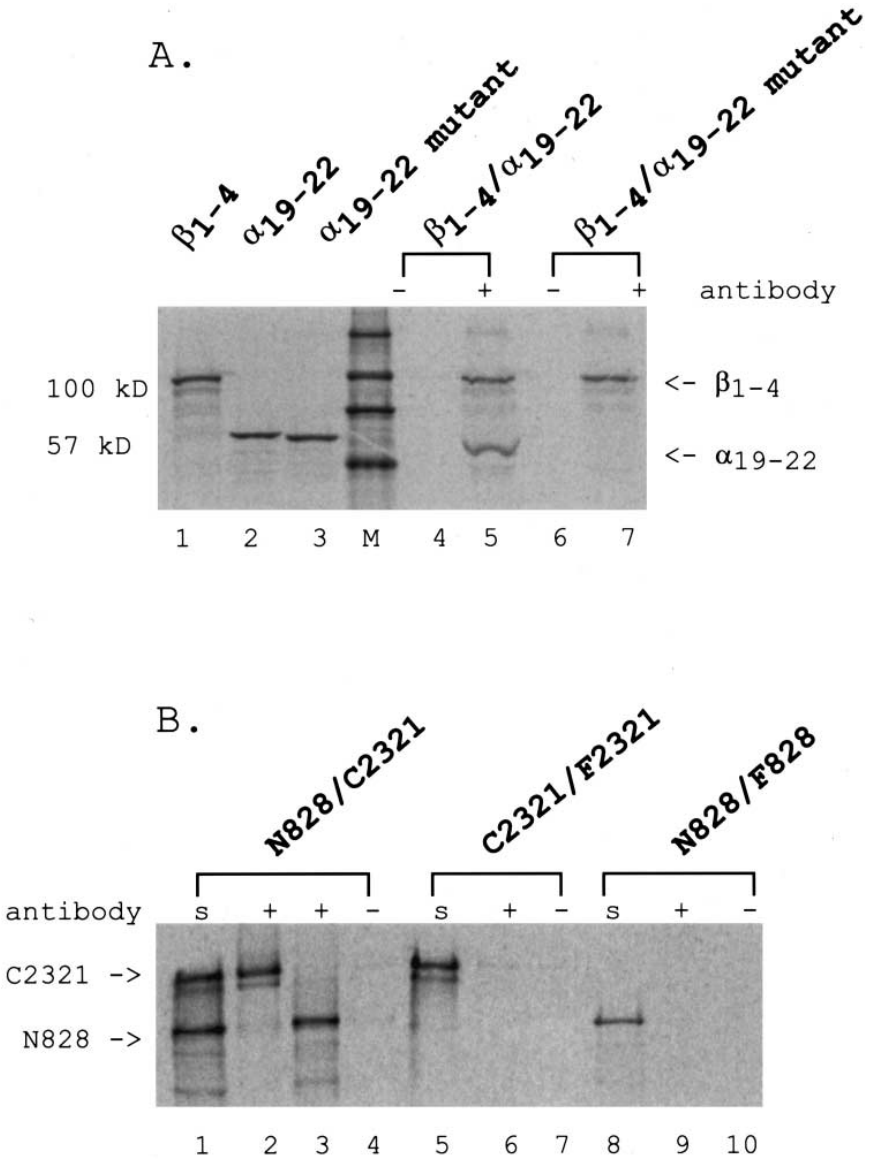

Fig. 3. A: Interaction between β_{1-4} spectrin and α_{19-22} spectrin in co-immunoprecipitation assay. In vitro translated product of β_{1-4} spectrin fragment (lane 1), α_{19-22} spectrin fragment (lane 2), α_{19-22} mutant spectrin fragment (lane 3). β_{1-4} was incubated with α_{19-22} in lanes 4 and 5. Similarly, β_{1-4} was incubated with α_{19-22} mutant in lanes 6 and 7. The absence or presence of antibody was indicated by (−) or (+), respectively. Lane M: ^{14}C-Methylated high molecular mass standards. B: Terminal domains of dystrophin do not interact with each other in co-immunoprecipitation assay. Lanes 1–4: ^{14}C-Labeled N828 and C2321 were incubated in the presence (+) or absence (−) of dys 2 (Novacastra, UK), an antibody directed against the C-terminus of dystrophin (lane 2) and dys 3 (Novacastra), an antibody directed against the N-terminus of dystrophin (lane 3). Lane 1: Supernatant(s) from both N828 and C2321. Lanes 5–7: ^{14}C-Labeled C2321 was incubated with its unlabeled flag version, F2321 in the presence (+) or absence (−) of an anti-flag antibody, M2. Lanes 7–9: ^{14}C-Labeled N828 was incubated with its unlabeled flag version, F828 in the presence (+) or absence (−) of the M2 antibody.

units and the entire C-terminus of dystrophin (Table 1). When both ^{14}C-labeled dystrophin fragments were incubated together with an antibody directed against the C-terminal dystrophin (dys 2), C2321 failed to precipitate N828 (Fig. 3B, lane 2). When the assay was reversed, N828 did not co-precipitate C2321 using a N-terminal specific dystrophin antibody (dys 3) as the detector (Fig. 3B, lane 3). Neither dystrophin fragments were precipitated in the absence of antibody (Fig. 3B, lane 4).

Interaction between the same terminal domains of dystrophin was then tested. In order to detect self-association of the same dystrophin fragment, a variant version of the original construct was designed. The resulting construct, F2321, encodes the exact dystrophin sequence as C2321, except with the addition of a flag-tag (MDYKDDDK) fusion polypeptide at its N-terminus which is recognized by a unique anti-flag antibody, M2 (Kodak, Rochester, NY). As shown in Fig. 3B, the unlabeled F2321 did not precipitate the ^{14}C-labeled C2321 with the anti-flag antibody (Fig. 3B, lane 6). The radiolabeled C2321 remained in the supernatant (Fig. 3B, lane 5) and was not precipitated in the absence of M2 antibody (Fig. 3B, lane 7). Similarly, no interaction was detected between the two N-terminal dystrophin fragments (Fig. 3B, lanes 8–10).

With the absence of evidence for interactions between the end-terminal domains, six additional overlapping clones were engineered to covered the entire α-helical rod domain of dystrophin (Table 1). Each of the eight dystrophin fragments was

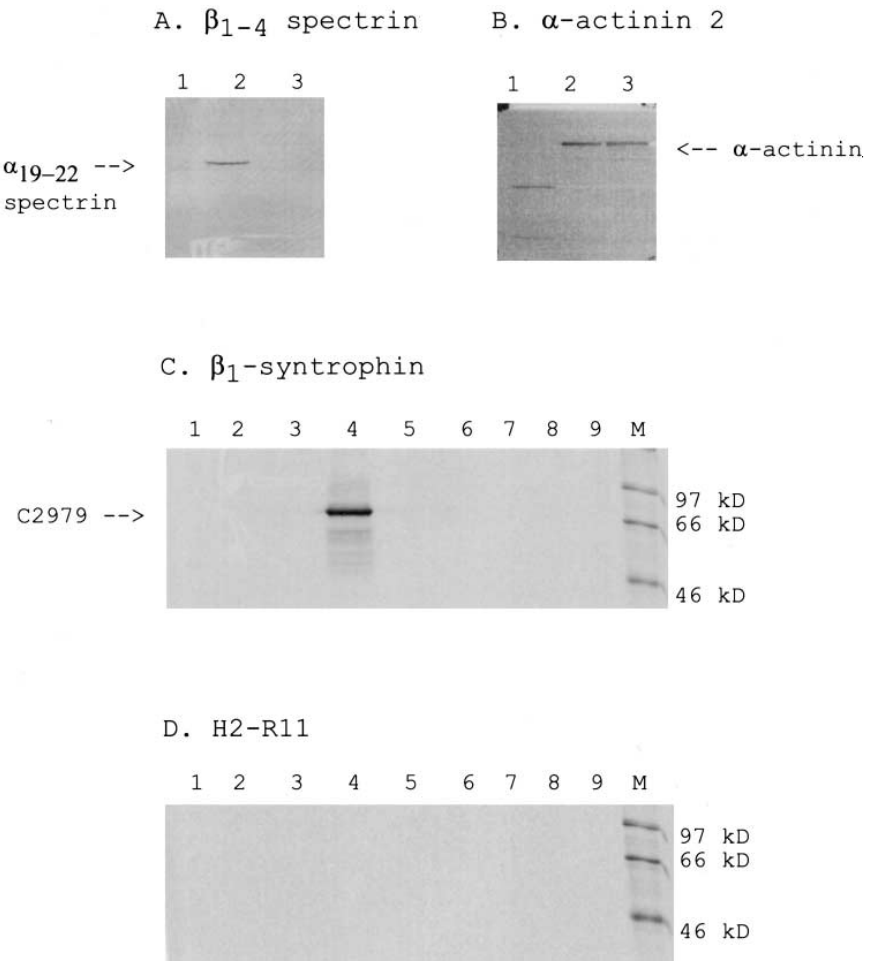

Fig. 4. In vitro translated dystrophin fragments do not interact with each other in blot overlay assay. A: [^{35}S]Methionine-labeled β_{1-4} spectrin was used as a probe. Lane 1: β_{1-4} spectrin; lane 2: α_{19-22} spectrin; lane 3: mutant α_{19-22} spectrin. B: [^{35}S]Methionine-labeled α-actinin 2 was used as a probe. Lane 1: N-terminal truncated α-actinin 2; lane 2: α-actinin 2; lane 3: α-actinin 3. C: [^{35}S]Methionine-labeled β_1-syntrophin was used as a probe. Lane 1: Mutant α_{19-22} spectrin; lane 2: α_{19-22} spectrin; lane 3: β_1-syntrophin; lane 4: C2979; lane 5: R19–H4; lane 6: R12–H3; lane 7: R7–R15; lane 8: H2–R11; lane 9: N828; lane M: ^{14}C-methylated high molecular mass standards. Note that β_1-syntrophin only bound to C2979 (lane 4). The result is consistent with the immunoprecipitation data in which among all the dystrophin fragments, only the C-terminus was able to interact with β_1-syntrophin (data not shown). D: [^{35}S]Methionine-labeled H2–R11 was used as a probe. The lanes were in same order as (C). Replica of the membrane were prepared and each membrane was overlaid with the other in vitro translated ^{35}S-labeled dystrophin fragments according to Section 2.

then tested for its ability to co-precipitate with each other, including itself. A total of 35 combinations were analyzed but no interaction was detected with any combination (data not shown).

3.3. *Absence of dystrophin self-association in blot overlay assays*

Blot overlay was subsequently used to investigate the potential binding between dystrophin fragments. Different in vitro translated dystrophin fragments were separated by SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was then blocked and incubated with the in vitro translated ^{35}S-labeled dystrophin fragments according to Section 2. To ensure all the dystrophin fragments were properly translated, the membrane was prepared in replica and analyzed by Western blot (data not shown). When ^{35}S-labeled β_{1-4} spectrin was used as a probe, it only bound to the wild-type α_{19-22} spectrin (Fig. 4A, lane 2) but not to itself (Fig. 4A, lane 1) or the mutant α_{19-22} spectrin (Fig. 4A, lane 3). Similarly, α-actinin 2 bound to both α-actinin 2 and 3 isoforms, as well as a N-terminal truncated α-actinin 2 (Fig. 4B). ^{35}S-Labeled β_1-syntrophin was also shown to bind to the C-terminal dystrophin fragment, C2979 (Fig. 4C, lane 4). However, none of the dystrophin fragments were able to bind to the membrane (Fig. 4D).

In addition, we also tested the ability of the in vitro translated dystrophin fragments to interact with full-length native dystrophin. The same set of in vitro translated ^{35}S-labeled dystrophin fragments were overlaid on nitrocellulose membranes containing tissue homogenates prepared from human skeletal muscle. Western blots, using dystrophin specific antibodies, showed the full-length dystrophin was intact (Fig. 5, lane 1). When ^{35}S-labeled β_1-syntrophin was used as a probe, it bound to the full-length dystrophin as expected (Fig. 5, lane 2). Similarly, when either β_{1-4} spectrin (Fig. 5, lane 6) or α-actinin 2 (Fig. 5, lane 7) were used as probes, they bound to spectrin and α-actinin at the expected size of 240 kDa (arrow) and 100 kDa (double arrow) respectively. Again, none of the dystrophin fragments were able to bind to the full-length dystrophin (Fig. 5, lanes 3–5).

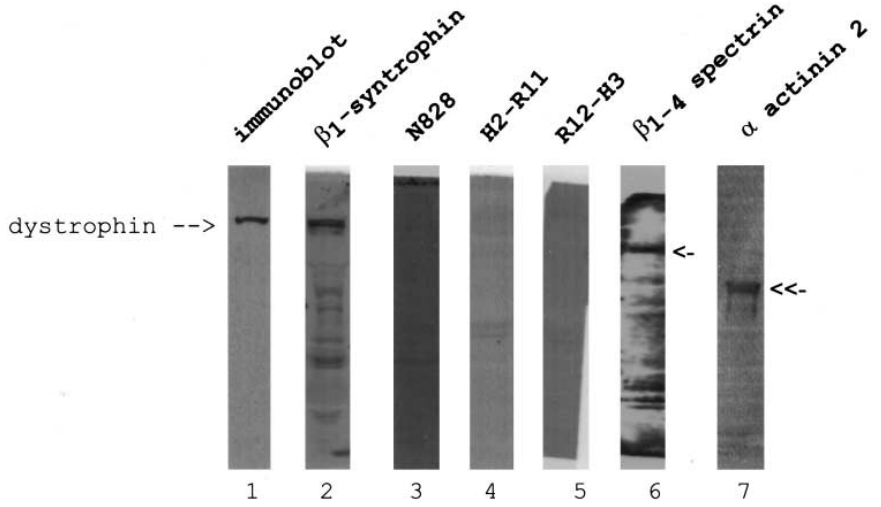

Fig. 5. In vitro translated dystrophin fragments do not interact with full-length dystrophin in blot overlay assay. Fifty micrograms of the muscle homogenate was loaded on 8.5% or 10% SDS-PAGE gel and transferred to nitrocellulose membrane. Replica of membrane were prepared and each membrane was overlaid with the in vitro translated ^{35}S-labeled dystrophin fragments according to Section 2. β_1-syntrophin, β_{1-4} spectrin and α-actinin 2 probe were used as positive controls. Lane 1: Western blot of the muscle extract using a C-terminal specific dystrophin antibody, dys 2. Lanes 2–7: Membrane was overlaid with different ^{35}S-labeled probes: β_1-syntrophin (lane 2); N828 (lane 3); H2–R11 (lane 4); R12–H3 (lane 5); β_{1-4} spectrin (lane 6); α-actinin 2 (lane 7).

4. Discussion

In summary, we expressed fragments of dystrophin that are about one-fourth of its full length by an in vitro translation system and tested their ability to interact with each other by immunoprecipitation and blot overlay experiments. Neither assay was able to identify any interaction between the in vitro translated dystrophin fragments (Table 2). In addition, none of the fragments interacted with the full-length native dystrophin from human muscle tissue homogenate in blot overlay assays. In contrast, when we expressed α-actinin or spectrin fragments that were homologous to the dystrophin fragments, we clearly showed a specific interaction between different isoforms of α-actinin as well as between spectrin fragments that contain the interchain binding sites. Collectively, these results suggested that dystrophin behaves quite differently in vitro from spectrin and α-actinin despite their significant homology. In support of this view, conformation and phasing analyses of the spectrin-like repeats in the dystrophin rod domain indicated an irregular and less rigid structure than spectrin [23,24]. Recently, Kahana et al. have also suggested that dystrophin might exist as a monomer based on the physical properties of the rod domain [25].

While both dystrophin monomers and dimers have been reported by electron microscopy [13–15], our findings supported the existence of dystrophin as a monomer. In a separate experiment, immunogold-labeling of muscle tissue with a C-terminal dystrophin antibody always localized dystrophin immediately beneath the muscle membrane [26] while an antibody raised against the N-terminal portion of the rod domain localized dystrophin at 15–20 nm internal to the sarcolemma [27]. Since neither antibody label showed any difference in relative distance to the membrane or periodicity in the distribution of the immunogold-label (which is approximately 125 nm, the predicted rod length of dystrophin), this finding was inconsistent with the current model of an anti-parallel dystrophin dimer but was supportive of a dystrophin monomer. (However, it did not rule out the possibility of a parallel dystrophin dimer.) In addition, there are examples of patients with large internal in-frame deletion in the rod domain but

Table 2
Summary of all possible combinations of interactions tested

	N828	H1–H2	H2–R11	R12–H3	R7–R15	R19–H4	C2321	C2979	β_1-syn	β_{1-4}	α_{19-22}	α-actinin
N828	–	–	–	–	–	–	–	–	–	–	–	–
H1–H2		ND	–	–	–	–	–	–	–	ND	ND	–
H2–R11			–	–	–	–	–	–	–	–	–	–
R12–H3				–	–	–	–	–	–	–	–	–
R7–R15					–	–	–	–	–	–	–	–
R19–H4						–	–	–	–	–	–	–
C2321							–	–	+	ND	ND	–
C2979								–	+	–	–	–
β_1-syn									–	ND	ND	–
β_{1-4}										–	+	ND
α_{19-22}											–	ND
α-actinin												+

Thirty-five different combinations from eight dystrophin fragments were tested for their ability to interact with each other. β_1-syntrophin, β_{1-4} spectrin, α_{19-22} spectrin and α-actinin were also included as positive controls. The presence or absence of interaction was indicated by + or –, respectively. ND, not determined. Note that the H1–H2 was not tested for its self-association because the domains encoded by N828 already included H1–H2.

nevertheless have very mild symptoms [28,29]. Such deletions are likely to be very disruptive had dystrophin existed as a dimer. Recently, Beckman has also argued against a dystrophin dimer because of the lack of dominant-negative effects in transgenic mice overexpressing truncated dystrophin [30].

Although our results suggested that dystrophin did not dimerize in vitro, we cannot rule out the possibility that dystrophin exists as a dimer in vivo. If this is the case, the interaction between dystrophin is likely to be weak as suggested by Pons and Fabbrizio who demonstrated that the majority of dystrophin prepared from chicken gizzard muscle and cardiac muscle was in monomeric form under electron microscopy [13,14,31]. On the other hand, it is possible that dystrophin might exist as a heterodimer with some unknown isoforms. Other factors, such as accessory proteins, phosphorylation or the specific conformation adopted by the full-length native dystrophin could also affect its self-association properties. Meanwhile, when we performed in vivo chemical crosslinking of dystrophin from mouse C2C12 myotubes, it often resulted in a large aggregate that was not interpretable, even after limited trypsin digestion (data not shown).

While we cannot unequivocally prove that dystrophin is a monomer, our results provide the strongest evidence today that dystrophin by itself, in the absence of accessory proteins, does not associate with itself in vitro. The current hypothesis that dystrophin is a dimer, which is based solely on its analogy to spectrin, needs to be re-examined more closely. Further studies, such as chemical crosslinking or sedimentation equilibrium analysis of in vivo purified dystrophin, should provide valuable information on the 'true state' of dystrophin.

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