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Binding sites involved in the interaction of actin with the N-terminal region of dystrophin

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Two actin-binding sites have been identified on human dystrophin by proton NMR spectroscopy of synthetic peptides corresponding to defined regions of the polypeptide sequence. These are Actin-Binding Site 1 (ABS1) located at residues 17–26 and Actin-Binding Site 2 (ABS2) in the region of residues 128–156. Using defined fragments of the actin amino acid sequence, ABS1 has been shown to bind to actin in the region represented by residues 83–117 and ABS2 to the C-terminal region represented by residues 350–375. These dystrophin-binding sites lie on the exposed domain in the actin filament.

Proton NMR; Dystrophin; F-actin; Interaction; α-Actinin; β-Spectrin

1. INTRODUCTION

Dystrophin, the protein that is absent or present in a modified form in Duchenne and Becker muscular dystrophies, respectively, is now established as a component of the cytoskeletal-membrane system of skeletal muscle. When isolated it has been shown to be a rod-like molecule of length 120–180 nm [1,2]. Current immunohistochemical studies indicate that it lies in a network with the spectrin-like domain lying parallel to and about 15 nm from the cytoplasmic face of the membrane and with the C-terminal region probably inserted in the membrane [3,4]. The function and location of dystrophin will depend on its interaction with the other components of the cytoskeletal-membrane system. Our earlier investigations to define these interactions have identified an actin-binding site on the N-terminal region of dystrophin encompassing residues 17–26 [5]. We designate this Actin-Binding Site 1 (ABS1). This short region of dystrophin shows strong homology (70% identity) with the amino acid sequence of the comparable regions of α-actinin [6] and β-spectrin [7]. When the common sequence Lys-Thr-Phe-Thr, which is part of ABS1, is aligned the homology extends further into all three molecules (Fig. 1). In view of this homology and the nature of the interaction of actin with α-actinin and β-spectrin it would be expected that further sites of interaction of actin on dystrophin exist. This has now been shown to be the case by extending our earlier studies using proton NMR spectroscopy of synthetic peptides corresponding to regions of the dystrophin molecule. In this communication we provide evidence for a second actin-binding site on dystrophin, Actin-Binding Site 2 (ABS 2), and identify the two sites on actin to which these regions of dystrophin bind.

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2. MATERIALS AND METHODS

Peptides were synthesised and purified as described previously [5]. They were checked for purity and composition by proton NMR. Actin was prepared as described by Levine et al. [8] and stored as the freeze-dried protein. Before use it was dissolved in 20 mM deuterated Tris-HCl, pH 7.9, containing 50 mM KCl, 2 mM MgCl, 0.5 mM DTT and polymerised by allowing to stand overnight at 4°C. The peptide corresponding to residues 83–117 of actin was isolated from the digest obtained after cyanogen bromide cleavage [8] and that corresponding to residues 350–375 synthesised. For spin labelling, 5 mg of peptide dissolved in 1.5 ml 6 M guanidine hydrochloride, 10 mM Tris-HCl, pH 8.0, was reduced overnight with a 20% molar excess of DTT. Excess DTT was removed by HPLC and the peak containing the peptide freeze-dried. The reduced peptide was immediately redissolved in the guanidine hydrochloride buffer, 5× molar excess of iodoacetimido-tempo (Sigma Chemical Co) added and left in the dark overnight at room temperature. Excess spin label was removed by HPLC and the labelled peptide freeze-dried and stored in the cold.

NMR spectroscopy was carried out on a Bruker 500MHz instrument as previously described [5]. Freeze-dried peptides were dissolved in ^2H_2O buffered with 20 mM deuterated Tris, pH 7.9. Titrations with actin were carried out by the addition of small aliquots of a stock solution of F-actin (200 μM).

3. RESULTS AND DISCUSSION

3.1. *Identification of Actin-Binding Site 2 (ABS2)*

In the course of screening synthetic peptides corresponding to regions downstream from ABS1 (residues

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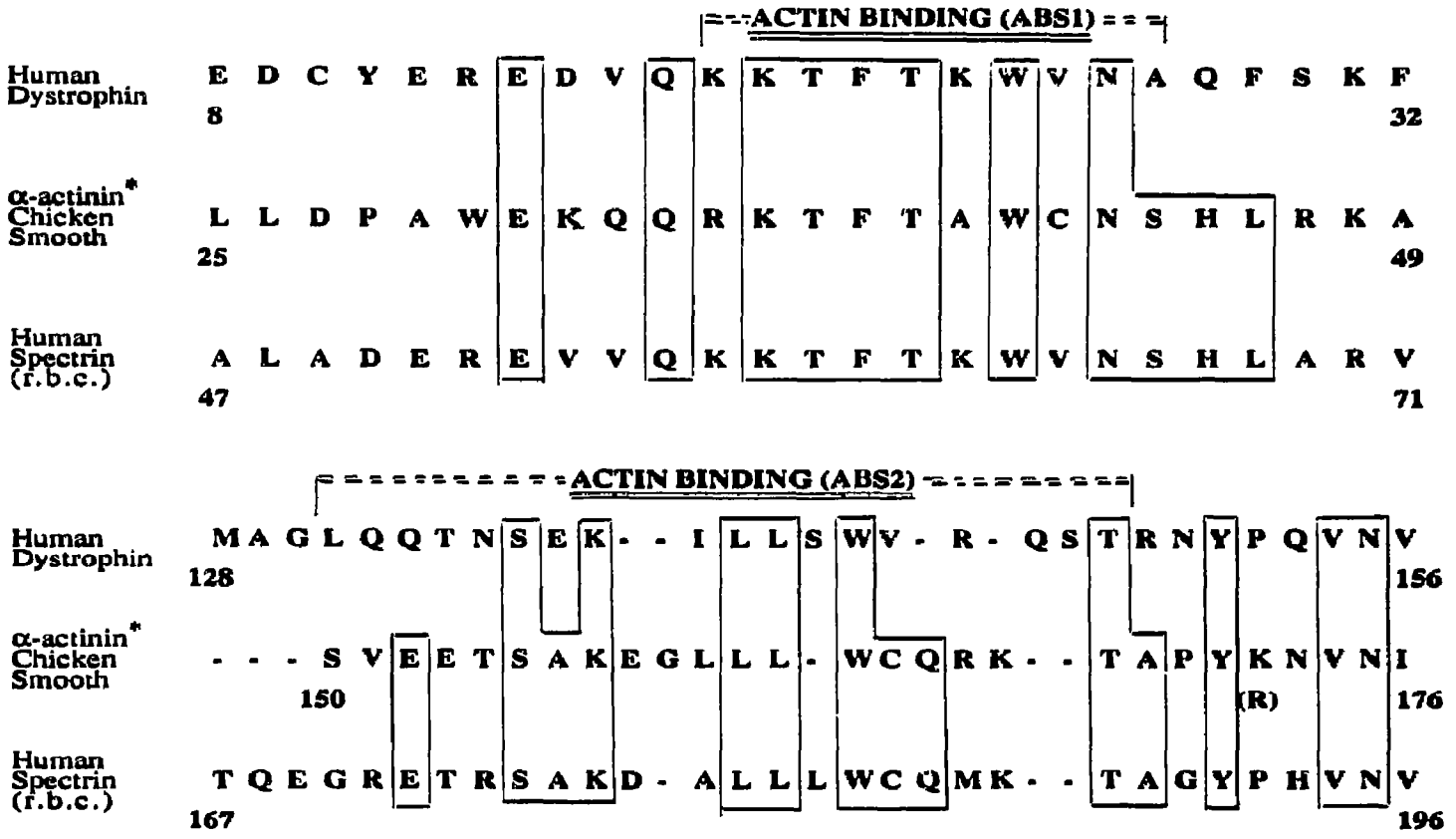

Fig. 1. Comparison of the amino acid sequences of the actin binding regions of human dystrophin (ABS1 and ABS2) with the corresponding segments of α-actinin [6] and β-spectrin [7]. *The corresponding sequence of chicken skeletal α-actinin is identical except at residue 172.

17–26) we observed a specific interaction with F-actin by a peptide consisting of residues 128–156. Titration of dystrophin peptide 128–156 with F-actin resulted in the selective broadening of the resonances of a number of residues e.g. the aromatic signals of Trp-143 and to a lesser extent those of Tyr-151 (Fig. 2). From the nature of these signals and those of other side chains that are progressively relaxed during titration, Gln γCH_2, Thr γCH_3, Asn βCH_2 and CH_3 of Leu, Val and/or Ile one can infer, given the primary sequence, that the central segment of the peptide (residues 131–148) is involved in complex formation with F-actin. This we designate Actin-Binding Site 2 (ABS2). In contrast, there was no evidence of F-actin binding to peptides consisting of residues 3429–3440 and 3656–3685 from the C-terminal region of dystrophin. The specific nature of the interaction with F-actin was also confirmed by the absence of detectable spectral effects, i.e. no evidence of binding ($K_b < 10^2$), when the peptide consisting of residues 128–156 and also the other actin-binding peptide, residues 10– 32, were titrated with bovine serum albumin.

3.2. *Site on actin interacting with Actin-Binding Site 1 (ABS1) of dystrophin*

To confirm that the dystrophin peptide, residues 128–156, contained a specific actin-binding site and to investigate the relation between ABS1 and ABS2, investigations were undertaken to identify the sites on actin that were involved in the interactions. Ideally for this approach peptides (100–500 μM) representing presumptive dystrophin-binding sites on actin should be titrated with dystrophin (0–100 μM). Under these conditions changes in the proton NMR spectrum of the actin peptides can readily be detected without spectral interference from the large dystrophin molecule. Unfortunately dystrophin is not yet available in the amounts required for this type of experiment and it is necessary to compromise by replacing dystrophin in such experiments with synthetic peptides representing its actin-binding sites. In some experiments, in order to distinguish the signal contributions from the actin and dystrophin peptides, aliquots were spin-labelled at unique cysteine residues where these occurred. The paramagnetic probe attached to one of the pair of peptides whose putative interaction is under investigation provides a sensitive read-out of the proximity of the probe to the interacting peptide. Spectral lines arising from amino acid side chains <1.5 nm from the probe broaden on complex formation with an isotropic distance dependence (r^{-6} from the spin label) indicating interaction has occurred [9]. The influence of the pa-

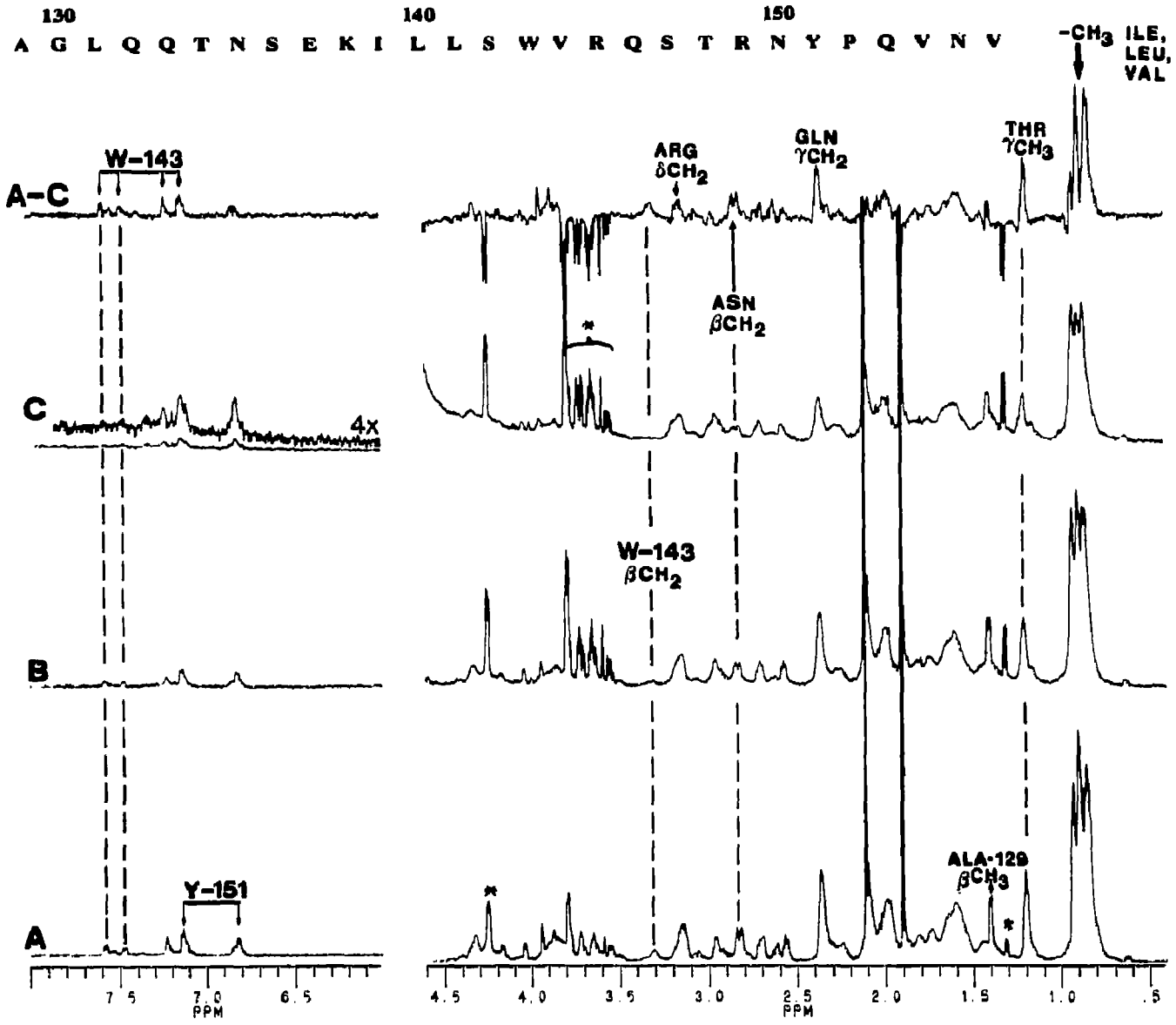

Fig. 2. Effect of F-actin on the proton NMR spectrum of human dystrophin peptide, residues 128–156. (A) 200 μM peptide, pH 7.9, T=300K. (B) and (C) spectra obtained on addition of 8 μM and 16 μM F-actin respectively, showing progressive relaxation of specific signals due to complex formation. These spectral perturbations are directly resolved by difference spectroscopy (A–C). Resonance assignments were obtained by homonuclear correlation experiments. Signals marked by * derive from low molecular weight impurities.

ramagnetic probe on resonances of the interacting peptide can be subsequently eliminated in situ by addition of a reducing agent. This provides an internal control for monitoring association between the peptides. In contrast insensitivity of the target peptide signals to the presence of the spin label during the titration provides confirmatory evidence that complex formation has not occurred.

A range of actin fragments consisting of synthetic peptides representing residues 1–18, 16–41, 29–58, 32–76, 96–117 and 350–375 as well as a peptide obtained by cyanogen bromide cleavage, residues 83–119, were tested for interaction with the dystrophin peptide consisting of residues 10–32 in which ABS1 is located. These actin peptides, derived from regions exposed on the actin filament, have previously been used by us in studies on the actin-binding regions of the regulatory proteins, troponin I and caldesmon, as well as those of myosin subfragment I. Titration with the dystrophin peptide, spin labelled on Cys-10, gave evidence of its interaction with the synthetic peptide corresponding to actin residues 96–117. The spectra illustrated in Fig. 3A

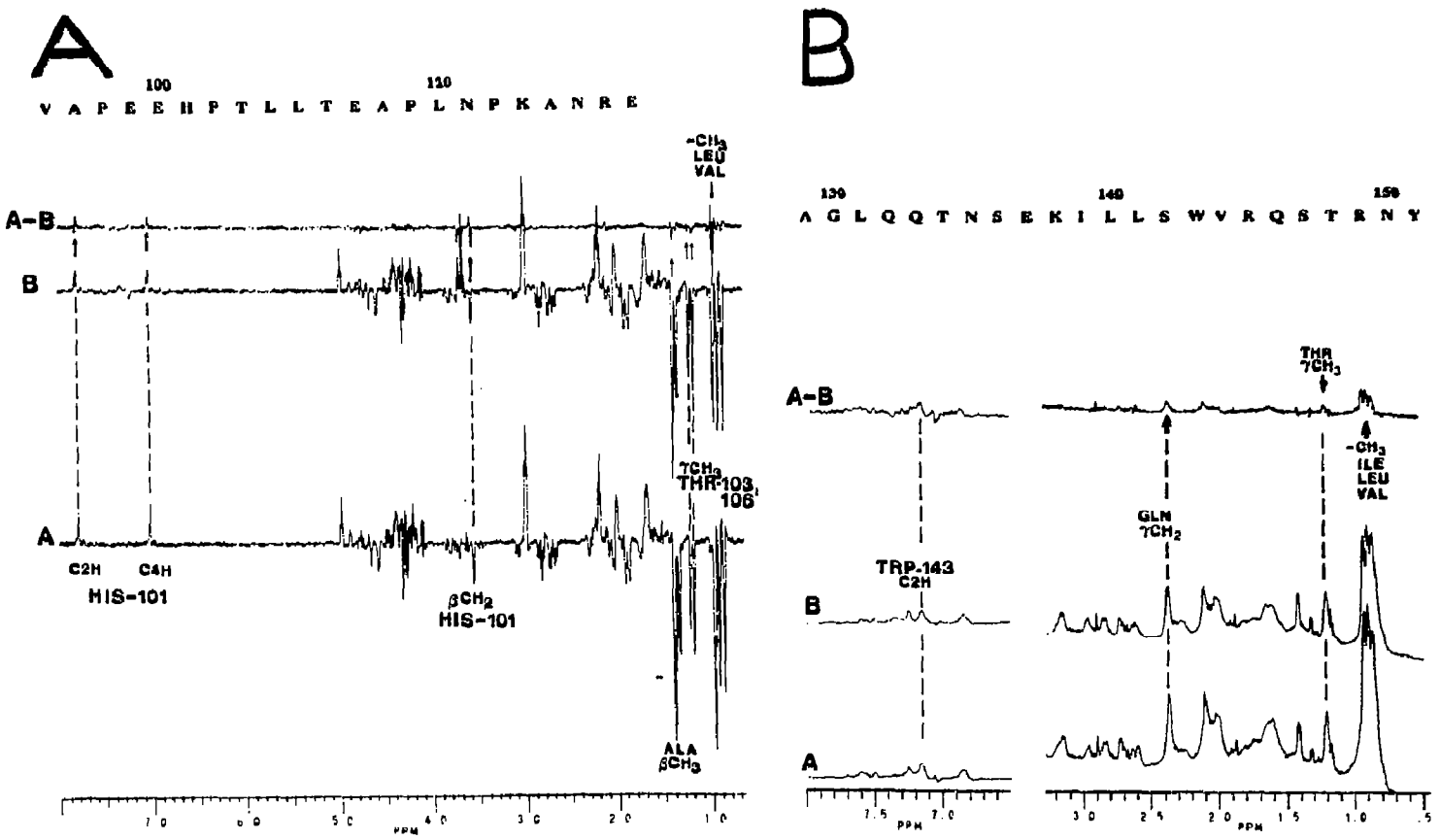

Fig. 3. (A) Interaction of dystrophin peptide, residues 10–32, containing ABS1 with actin peptide corresponding to residues 96–117. Two pulse spin echo spectrum (τ=60 msec) resolving signals on the basis of J-coupling (e.g. [8]). Titration of actin peptide residues 96–117 (480 μM) with dystrophin peptide residues 10–32, spin-labelled at Cys-10 (20 μM, upper trace) resulted in specific paramagnetic broadening on the signals shown. These distance-dependent effects imply that the molecules interact in a head to tail fashion. (B) Changes in the proton NMR spectrum of dystrophin peptide residues 128–156 when titrated with actin peptide residues, 350–375, spin-labelled at Cys-374. (A) 120 μM dystrophin peptide alone. (B) 120 μM dystrophin peptide with addition of 16 μM actin peptide results in the broadening of signals (difference spectrum, A–B) homologous to those observed in interaction with F-actin (see Fig. 2). The signals detected (A–B) occur as a consequence both of the proximity of the spin label and of complex formation alone.

show that the probe on the dystrophin peptide is located in the vicinity of His-101 of the actin molecule and the side chain(s) of leucine (Leu-104, -105 and/or -110) and threonine (Thr-103 and -106). Similar results were obtained on titrating the same spin labelled dystrophin peptide with the cyanogen bromide fragment of actin, residues 83–119 while on addition of 1.0 mM DTT to the peptide mixture the paramagnetic effects were eliminated. None of the other synthetic peptides or cyanogen bromide fragments of actin exhibited evidence of binding to dystrophin peptide residues 10–32. It was therefore concluded that ABS1 of dystrophin interacts with part of the region on actin represented by residues 83–117.

3.3. *Site on actin interacting with Actin-Binding Site 2 (ABS2) of dystrophin*

The spectral changes observed on the interaction of dystrophin peptide residues 128–156 with F-actin were found to be reproducible upon titration with the C-terminal actin peptide consisting of residues 350–375. This result was confirmed using the actin peptide spin labelled at Cys-374. It can be seen from Fig. 3B that during titration the residues perturbed by the spin label on the actin peptide are those of glutamine, threonine and the aromatic and aliphatic hydrophobic amino acids of the dystrophin peptide. These are exactly the same residues that are perturbed when the same dystrophin peptide interacts with F-actin (compare Fig. 2). There was no evidence of dystrophin peptide residues 128–156 binding at the other dystrophin binding site on actin, residues 83–119, or interacting with any other of the actin peptides in the library. We therefore conclude that ABS2 of dystrophin binds to the C-terminal region of actin represented by residues 350–375.

The two sites involved in binding dystrophin are located on regions of the actin molecule that are exposed in the F-actin filament [10]. The residues on dystrophin involved in these interactions with actin are homologous with comparable regions of the α-actinin and β-spectrin molecules (Fig. 1). In the light of this conclu-

sion it is significant that antibody and cross-linking experiments have suggested that two sites on α-actinin may be involved in actin binding [11,12]. The regions of the actin molecule implicated in these studies are those which contain the sites shown directly by the proton NMR studies to be involved in the binding of dystrophin. In view of the sequence homology in the N-terminal regions of α-actinin and β-spectrin it seems likely that the same sites on actin i.e. those in the regions of residues 83–117 and 355–370, have the capacity to bind dystrophin, α-actinin or β-spectrin. If this is the case it raises the question as to which of these proteins preferentially binds to actin under conditions in which there is not enough actin to saturate all.

Although our investigations indicate that the two sites, ABS1 and ABS2, are independently identifiable it is not yet possible to decide at this stage if, in the intact molecule, they are in fact close together and form part of a larger contact region. In this context it is important to determine whether these two sites on a given dystrophin molecule bind to the sites on one actin subunit. From the recently published three dimensional model of the actin molecule [10] it appears that although the dystrophin binding sites on actin are fairly close (1–1.5 nm) they do not lie on the same side of the outer domain. It is of interest that interaction of actin with the myosin head and the regulatory proteins also involves multiple sites on the outer domain of the molecule in F-actin [13] and the binding of dystrophin to actin may involve further docking sites. The data available suggests that the arrangement of the sites on both proteins may enable one dystrophin molecule to interact with an actin monomer in each of the two strands in the F-actin filament. In this manner the two proteins would be rigidly linked in the correct conformation. An aspect of our current research is directed to the resolution of this problem.

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