In vitro interactions of *Caenorhabditis elegans* dystrophin with dystrobrevin and syntrophin

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Abstract Dystrophin, the product of the gene mutated in Duchenne muscular dystrophy (DMD) is bound by its C-terminus to a protein complex including the related protein dystrobrevin. Both proteins contain a putative coiled-coil domain consisting of two α-helices. It has been reported that the two proteins bind to each other by the first one of the two α-helices. We have revisited this question using the *Caenorhabditis elegans* homologs of dystrophin and dystrobrevin. In vitro interaction occurs through the more conserved second helix. We propose a new model of dystrophin interactions with associated proteins.

Key words: Dystrophin; Dystrobrevin; Syntrophin; Duchenne muscular dystrophy; Nematode

1. Introduction

Duchenne and Becker muscular dystrophies (DMD and BMD) are allelic forms of progressive myopathies caused by the disruption of the dystrophin gene. This gene encodes a 3685-amino acid protein found in skeletal and cardiac muscles and in the nervous system [1,2]. The function of the dystrophin protein is not clearly established yet. Dystrophin is present under the sarcolemma of skeletal muscles, where it binds cortical actin via its N-terminal region. Through its C-terminal region, dystrophin binds to a complex of membrane proteins termed the dystrophin-associated proteins (DAPs) (reviewed in [3]).

Dystrobrevins form a family of dystrophin-associated and dystrophin-related proteins [4,5]. They are of particular interest because the first member of the family was originally identified as a copurification product of nicotinic acetylcholine receptors isolated from *Torpedo californica* electric organs [4]. The structural similarity between dystrophin and dystrobrevin extends to two α-helices located near the C-terminus of each protein and predicted to form coiled-coil protein interaction domains. Previous studies have shown that these domains are sufficient to mediate the physical interaction between the two proteins and that this interaction likely takes place between the first helices (H1) of each protein [6]. The protein region directly upstream of H1 in both dystrophin and dystrobrevin long isoforms is thought to associate with syntrophins [6,7].

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The *Caenorhabditis elegans* genome contains single homologs of dystrophin, dystrobrevin and syntrophins, named dys-1, dyb-1 and F30A10.8, respectively [8,9]. In this study, we analysed in vitro interactions between the *C. elegans* proteins and show that they differ from the model established in mammals.

2. Materials and methods

GST alone and GST fusion proteins were produced in *Escherichia coli* using the pGEX vectors (Pharmacia). Clone BB810 contains DYS-1 amino acids 3402–3674. Clone AN450 contains DYB-1 amino acids 390–543. These clones were obtained by PCR and their sequence verified after cloning. Deletion derivatives were obtained by directed (clones ΔNS and ΔNDΔBS) or non-directed (random) procedures using appropriate restriction and exonuclease enzymes. Deletions generated by exonucleases were sequenced and only clones in which the reading frame was restored were conserved. Fusion proteins were produced in 50 ml *E. coli* cultures. After cell sonication and centrifugation, the supernatant was loaded on glutathione-Sepharose beads (Pharmacia). Approximately 20 μg of resin bound proteins were washed in binding buffer (HEPES 20 mM pH 7.4, KOAc 110 mM, NaOAc 5 mM, $Mg(OAc)_2$ 2.5 mM, NP40 0.05%, DTT 1 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml, pepstatin 10 μg/ml, PMSF 1 mM). ^{35}S-labelled DYS-1, DYB-1 or F30A10.8 was synthesised using a coupled in vitro transcription and translation kit (Promega) using cDNAs yk12c11, yk118e3 or yk109h11 as a template. Incubation was performed for 2 h at 4°C. GST controls were performed using 1–2 times the amount of fusion proteins. Following 5 washes with binding buffer, labelled proteins were eluted by boiling for 3 min in gel loading buffer. Gels were exposed overnight and revealed using a radiographic analyser (Fuji BAS-1500). Band intensity was quantitated using the machine software in PSL/mm^2. To normalise data from different experiments, binding intensity was expressed as the ratio (sample x/GST). Each sample was assayed between three and eight times.

3. Results

3.1. Alignment of human and nematode sequences of dystrophin and dystrobrevin

We previously reported the identification of a dystrophin homolog (DYS-1) and a dystrobrevin homolog (DYB-1) in the *C. elegans* genome [8,9]. The *C. elegans* proteins contain the same structural features as their mammalian counterparts, suggesting that their physiological function and partners have been conserved through evolution. The homology includes the two α-helices forming the putative coiled-coil domain. However, the precise alignment between human and nematodes amino acid sequences from this region surprisingly reveals a much higher conservation for helix 2 (52–53%) than for helix 1 (25–28%) for both dystrophin and dystrobrevin (Fig. 1). This observation was puzzling, since a high degree of conservation is generally considered to carry a conservation of function. We therefore investigated the question of dystrophin-dystrobrevin association in *C. elegans*.

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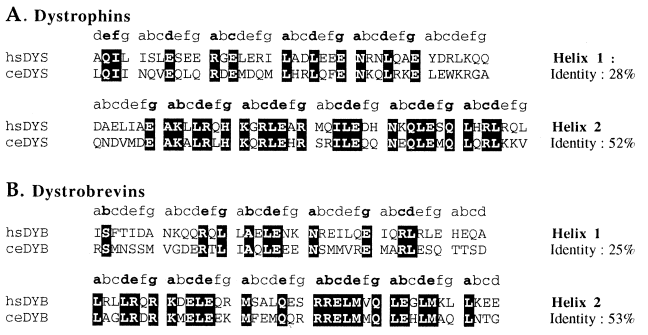

Fig. 1. Alignment of human and *C. elegans* dystrophins and dystrobrevins. Region of the putative α-helices forming the predicted coiled-coil domain. Identical residues are decorated in black. hsDYS: human dystrophin; ceDYS: *C. elegans* dystrophin; hsDYB: human dystrobrevin α1; ceDYB: *C. elegans* dystrobrevin. The letters a–g designate the positions of residues within the heptad of the coiled-coil domain.

3.2. Determination of DYS-1 and DYB-1 sequences necessary for in vitro association

We tested the in vitro association of dystrophin (DYS-1) and dystrobrevin (DYB-1) by incubation of in vitro translated ^{35}S-radiolabelled proteins with affinity purified GST fusion proteins (Fig. 4). GST alone was used as a negative control in all cases. DYS-1 and DYB-1 fragments produced as fusion proteins were 273-amino acid and 154-amino acid fragments encompassing the coiled-coil domains. GST-DYS-1 (BB810) and GST-DYB-1 (AN450) consistently retained ^{35}S-radiolabelled DYB-1 and DYS-1 respectively, thus demonstrating that *C. elegans* dystrophin and dystrobrevin associate in vitro. These combinations were used as positive controls in all subsequent experiments.

To determine the regions necessary for this interaction, we generated deletions derivatives of both GST-DYS-1 (BB810) and GST-DYB-1 (AN450). The structure of the deletions is shown on Fig. 2. We then assayed the ability of the deleted forms to bind to the ^{35}S-radiolabelled proteins.

GST-DYS-1 deletions ΔNS and 8′, removing parts of the region downstream of helix 2, did not alter binding to in vitro translated ^{35}S-radiolabelled DYB-1 (Fig. 3A). Deletion 15, removing the entirety of helix 1 of DYS-1, did not alter binding to DYB-1, indicating that helix 1 is not necessary for this interaction. However, deletion 11′, in which most of helix 2 is removed, displays a dramatically reduced binding to DYB-1 (Fig. 3A).

In the opposite set of experiments, GST-DYB-1 deletions 3′3 and 6′4 were able to bind ^{35}S-radiolabelled DYS-1 as well

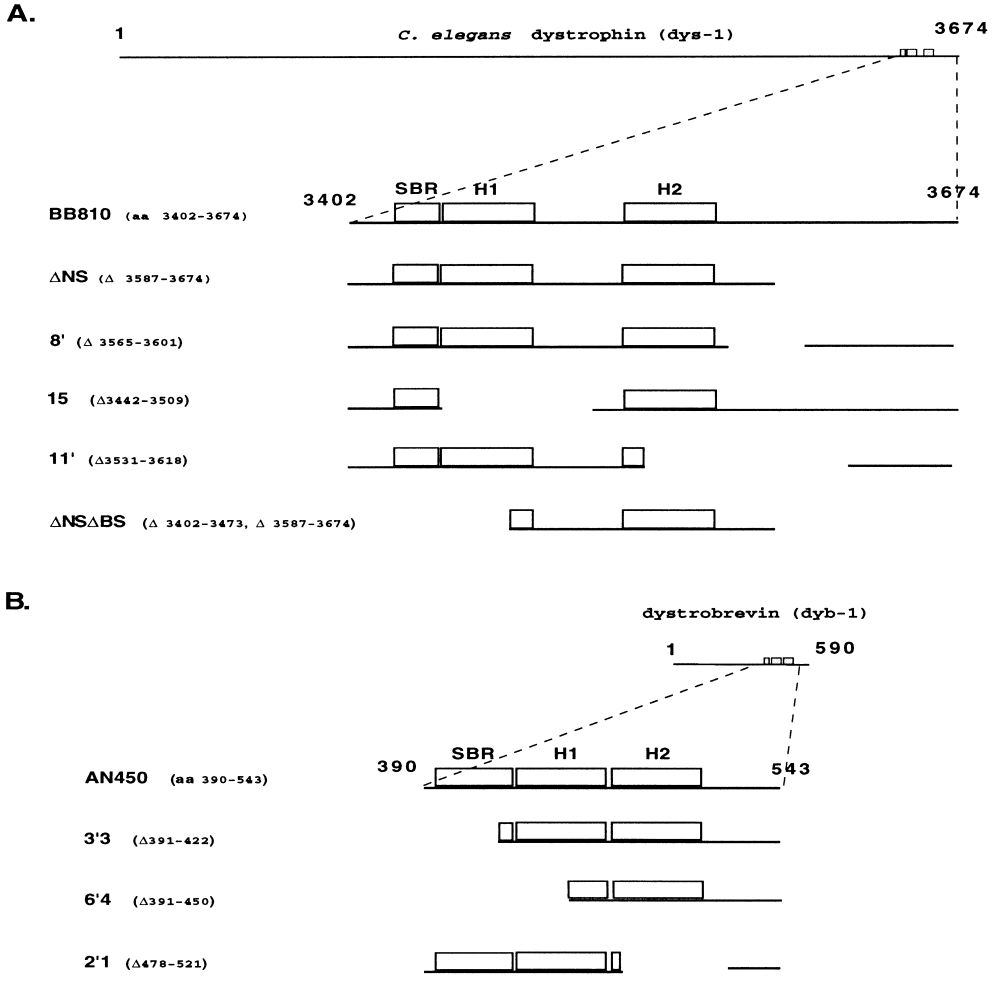

Fig. 2. Schematic representation of the GST-DYS-1 (A) and GST-DYB-1 (B) fusion proteins used in the study. The boxes indicate the α-helices forming the predicted coiled-coil domains. The top line shows the position of the coiled-coil domains in the protein. The region included in the GST fusions is enlarged below. SBR: Syntrophin-binding region; H1: helix 1; H2: helix 2.

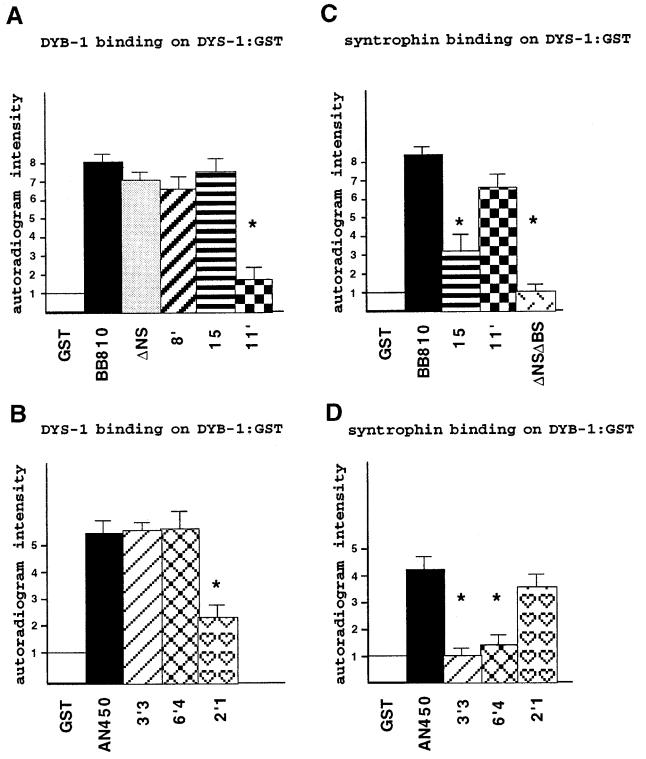

Fig. 3. Binding properties of GST-DYS-1 (A) and GST-DYB-1 (B) and deletions derivatives. A: ^{35}S-radiolabelled DYB-1 was incubated with GST-DYS-1 (BB810) and deletion derivatives. B: ^{35}S-radiolabelled DYS-1 was incubated with GST-DYB-1 (AN450) and deletion derivatives. C and D: ^{35}S-radiolabelled F30A10.8 (syntrophin) was incubated with the same proteins as in A and B, respectively. Binding is expressed in relative units. One unit is defined as the amount of signal detected on the GST (negative control) lane. Scale is linear. Asterisks indicate values significantly different from wild-type (either BB810 or AN450).

as the original clone AN450 (Fig. 3B). Inversely, deletion 2′1, in which most of helix 2 is missing, displays a reduced binding to DYS-1 (Fig. 3B). The residual affinity of clone 2′1 for DYS-1 may be explained by the presence of the first heptad of the second helix in this deletion. Alternatively, the C-terminal part of helix 1 of DYB-1 may play a role in this interaction.

These results show that, for DYS-1 as well as for DYB-1, the second helix (H2) seems to be the critical region for DYS-1-DYB-1 interaction, whereas the first helix (H1) appears dispensable.

3.3. Determination of DYS-1 and DYB-1 sequences necessary for binding to F30A10.8

Since we had generated the clones and deletion derivatives mentioned above, we used them to determine the regions necessary for binding of the *C. elegans* syntrophin homolog F30A10.8. As a prerequisite, we confirmed that the *C. elegans* syntrophin binds to both DYS-1 and DYB-1, and that binding regions were located in the vicinity of the putative coiled-coil domain by showing that BB810 and AN450 were able to retain ^{35}S-radiolabelled F30A10.8. Deletions derivatives were subsequently tested. GST-DYS-1 deletion derivative 15 showed a strong reduction of F30A10.8 binding, indicating that structural determinant in or very close to helix 1 are critical for this interaction. We then engineered an additional deletion, ΔNSΔBS, lacking the first half of helix 1 plus the upstream flanking region. As expected, this clone does not bind F30A10.8 at all (Fig. 3C).

GST-DYB-1 binding to F30A10.8 was reduced to GST levels in clone 3′3, lacking the first 32 amino acids of AN450, thus showing that removal of this region abolishes syntrophin binding to GST-DYB-1 (Fig. 3D).

4. Discussion

In this paper, we used an in vitro assay to show that the *C. elegans* homologs of dystrophin (DYS-1) and dystrobrevin (DYB-1) associate to each other and to syntrophin. We further analysed the binding determinants of these interactions by a deletion study. This study was triggered by the observation that sequence homologies between the mammalian and nematode proteins vary greatly along the putative coiled-coil domain thought to mediate this interaction in mammals. These variations did not fit with the proposed functional domains identified in mammals.

The binding experiments we carried on show that *C. elegans* dystrophin and dystrobrevin interact in vitro through the putative coiled-coil domain. Moreover, deletion experiments reveal that the second helix (H2) of this domain is more critical than the first one for DYS-1-DYB-1 interaction. This is consistent with the sequence alignment between nematode and mammalian forms of the proteins, which is higher for helix 2 than for helix 1. Not only is the second helix of each protein more conserved than the first one, but it also forms a more canonical coiled-coil domain [10]. Our results differ from those reported by Sadoulet-Puccio et al. with mammalian proteins [6]. The authors identified dystrophin clones in a two-hybrid search for proteins interacting with the C-terminal end of dystrobrevin. They tentatively mapped the domain of interaction to the first helix (H1) by using deleted forms in the two-hybrid assay. It is possible that the very short stretches of amino acids used in some constructs did not fold properly and failed to reveal a positive role for helix 2. The alternative hypothesis is that mammalian and nematode proteins do not associate through the same determinants.

Binding to the syntrophin homolog F30A10.8 is consistent with previously published observations. Our results obtained

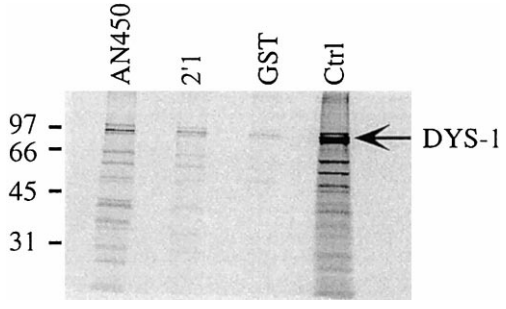

Fig. 4. Representative example of SDS-PAGE autoradiograms showing in vitro interactions between DYS-1 and DYB-1. Left three lanes: elution product of beads loaded with GST-DYB-1 AN450 (amino acids 390–543), deletion derivative 2′1 (lacking amino acids 478–521), GST alone and after incubation with equal amounts of in vitro translated ^{35}S-radiolabelled DYS-1 (amino acids 2857–3674), respectively. Ctrl: aliquot of ^{35}S-radiolabelled DYS-1. Images and quantification were obtained using a radiographic analyser (Fuji BAS-1500). Numbers on the left indicate molecular weight.

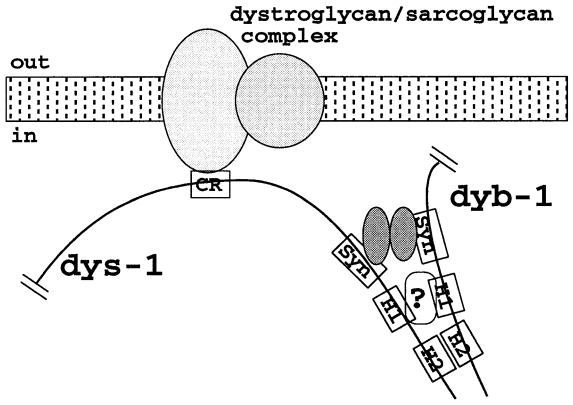

Fig. 5. Working model showing proposed molecular interactions of dystrophin with associated proteins in *C. elegans*. Dystrophin (dys-1) associates with dystrobrevin (dyb-1) by the second helix of their predicted coiled-coil domain (H2). Both dys-1 and dyb-1 are able to bind syntrophin by a region (Syn) located upstream of the first helix (H1). By analogy to mammalian models, two syntrophins (dark ellipses) have been represented, although this has not been documented in *C. elegans*. Dystrophin is thought to be associated with the transmembrane dystroglycan/sarcoglycan complex by its cysteine-rich (CR) region. Helix H1 of dys-1 and dyb-1 may be engaged in protein-protein interactions with an unidentified partner.

with both DYS-1 and DYB-1 point to a region upstream of helix 1. Results obtained with dystrophin clone 15 may also reveal a role for helix 1 of DYS-1, since this clone does not bind to syntrophin as well as wild-type does. However, it is also possible that the large helix-1 deletion immediately adjacent to the short syntrophin-binding region (SBR) modifies the conformation of the latter. Comparison of the *C. elegans* and mammalian dystrobrevin sequences reveal the presence of a highly conserved motif (amino acids 400–420 of DYB-1) in this region [9]. This motif is deleted in clone 3′3, unable to bind F30A10.8. It is therefore likely that this conserved motif carries structural determinants crucial for syntrophin binding. Although our data point to the corresponding region for DYS-1-F30A10.8 interaction, this region is less conserved between DYS-1 and its mammalian counterparts, except for a short proline-rich region immediately adjacent to helix 1 [8].

Finally, we propose a working model for dystrophin interactions with its partners in *C. elegans*. This model is a variation of published models [5,6] integrating the data from the present study (Fig. 5). In this model, dystrophin and dystrobrevin interact physically through the second helix of the coiled-coil domain. They may also interact indirectly through the syntrophins bound to their respective syntrophin binding regions. In mammals, dystrophin also interacts with the transmembrane dystroglycan-sarcoglycan complex by its cysteine-rich region (CR). The lack of biochemical data renders this part of the model speculative at the moment in nematodes. However, this model is supported by the presence of homologs for several members of the dystrophin complex in the *C. elegans* genome.

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