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# Troponin T is capable of binding dystrophin via a leucine zipper

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**Abstract** Using genetic and physical assays for protein–protein interactions, we identified a fast isoform of troponin T that binds to dystrophin. Troponin T specifically bound to the first of two highly conserved leucine zipper motifs in the carboxy terminus of dystrophin [1,2]. Single amino acid changes in the zipper predicted to disrupt α-helix formation or cause steric hindrance abolished this binding. These data support the hypothesis that dystrophin couples the contractile apparatus to the sarcolemma and indicate that leucine zipper mediated protein–protein interactions are functionally important in the cytoskeleton as well as the nucleus.

*Key words:* Duchenne muscular dystrophy; Dystrophin; Troponin; Protein–protein interaction; Cytoskeleton

## 1. Introduction

Duchenne muscular dystrophy is a severe X-linked recessive myopathy caused by defects in the gene encoding dystrophin, a 427 kDa actin-binding protein [3] that is related in sequence and structure to spectrin and α-actinin [4,5]. In skeletal muscle, it is associated with the cell membrane (sarcolemma) [6–8]. While the precise function of dystrophin is unknown, its absence leads to several pathophysiological changes that ultimately result in profound necrosis. Biochemical studies have led to the identification of a dystrophin–glycoprotein complex (DGC) that links the sarcolemma and extracellular matrix to the carboxy terminus of dystrophin [9,10,11,12]. Interestingly, this region of dystrophin contains two leucine zipper motifs separated by a 44 amino acid proline rich spacer [2]. The first of these motifs is identical in human, mouse, and chicken dystrophin and conserved in both *torpedo* dystrophin and utrophin, a dystrophin related protein. The second zipper is identical in all of these proteins. As the leucine zipper is a well-characterized mediator of protein–protein interactions, we hypothesized that they were sites of dystrophin complex assembly. Using the two-hybrid system [14,16] to probe these interactions, we identified a novel association between dystrophin and a fast isoform of troponin T. Notably, troponin T is the first skeletal muscle specific protein demonstrated to bind dystrophin. This physical association suggests a functional link between the cell membrane and the contractile apparatus.

## 2. Materials and methods

### 2.1. Two-hybrid system library construction

Poly(A)$^+$ RNA was purified from adult C57Bl/10J mouse hindlimb skeletal muscle RNA using Poly A Quik columns (Stratagene, La Jolla, CA). cDNA was synthesized with SuperScript reverse transcriptase (Gibco/BRL, Gaithersburg, MD) and cloned essentially as described in [16], except that λACT was digested with *Xho*I and a single T filled in with the Klenow fragment of DNA polymerase I instead of Taq polymerase. 0.2 μg of cDNA was ligated to 2 μg of vector and packaged with Gigapack Gold II (Stratagene, La Jolla, CA). Two million primary recombinant plaque forming units (pfus) were obtained. The library was amplified and excised in plasmid form by in vivo recombination in *E. coli* host BNN132.

### 2.2. Yeast plate lifts

Yeast colonies were grown to approximately 1 mm diameter and lifted onto Hybond N filters (Amersham, Buckinghamshire, UK). Filters were immersed in liquid nitrogen for 5 s and allowed to dry at room temperature, then layered on 0.5 ml of 1 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactoside (X-gal) in Z buffer and incubated for 30–90 min at room temperature. Z buffer is 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.25 g $MgSO_4 \cdot 7H_20$, and 2.7 ml β-mercaptoethanol per liter [17].

### 2.3. Quantitative β-galactosidase assays

Dystrophin carboxy terminus encoding fragments were generated by the polymerase chain reaction (PCR) and cloned into the *Eco*RI site of pMA424. Constructs have the following extents: $DysH_4L_aL_b$ (aa: 2,925–3,586); $DysH_4L_a\Delta_b$ (aa: 2,925–3,520); $DysH_4\Delta_a\Delta_b$ (aa: 2,925–3,498); $DysH_4L_{Ile}\Delta_b$ (aa: 2,925–3,520); $DysH_4L_{Pro}\Delta_b$ (aa: 2,925–3,520). Transformations were performed with two independent isolates, one of which was sequenced in its entirety and translated in vitro. β-galactosidase activity is expressed in Miller units [17] and is the average of 5 independent transformants with standard errors less than 20%.

### 2.4. In vitro translation and protein binding assays

Recombinant dystrophin protein fragments were produced in *E. coli* using the pRSET expression system (Invitrogen, San Diego, CA). Overnight cultures were pelleted and resuspended in sonication buffer (50 mM sodium phosphate pH 8.0, 100 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.2% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), subjected to one freeze-thaw cycle, and sonicated for 1 min at a medium setting. Lysates were clarified by centrifugation (10 min, 10,000 × *g*), and the supernatants were chromatographed on an NTA-nickel affinity column (Qiagen, Chatsworth, CA). Proteins were eluted with 100 mM HEPES pH 7.5 and 50 mM EDTA. Protein concentrations were determined quantitatively by the method of Bradford [27] and qualitatively by Coomassie blue staining. 2 μg of protein was fractionated on non-denaturing 10% polyacrylamide gels containing 0.1% Triton X-100 and 375 mM Tris·HCl pH 8.8 and transferred to nitrocellulose. Blots were blocked in binding buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100) supplemented with 5% non-fat dry milk. To generate probes for for in vitro binding, the full-length mouse troponin T and ARBP cDNAs were cloned into pRSET C and used to program a TNT rabbit reticulocyte lysate in the presence of $^{35}$S-L-methionine as described by the vendor (Promega, Madison, WI). 1 μl was fractionated by 10% SDS-PAGE and visualized by autoradiography. Reticulocyte lysate containing the radiolabelled probe was diluted 1:50 in binding buffer supplemented with 1% milk and incubated with the blot overnight at 4°C. Blots were subjected to 5 ten minute washes in binding buffer and exposed for 1–5 h.

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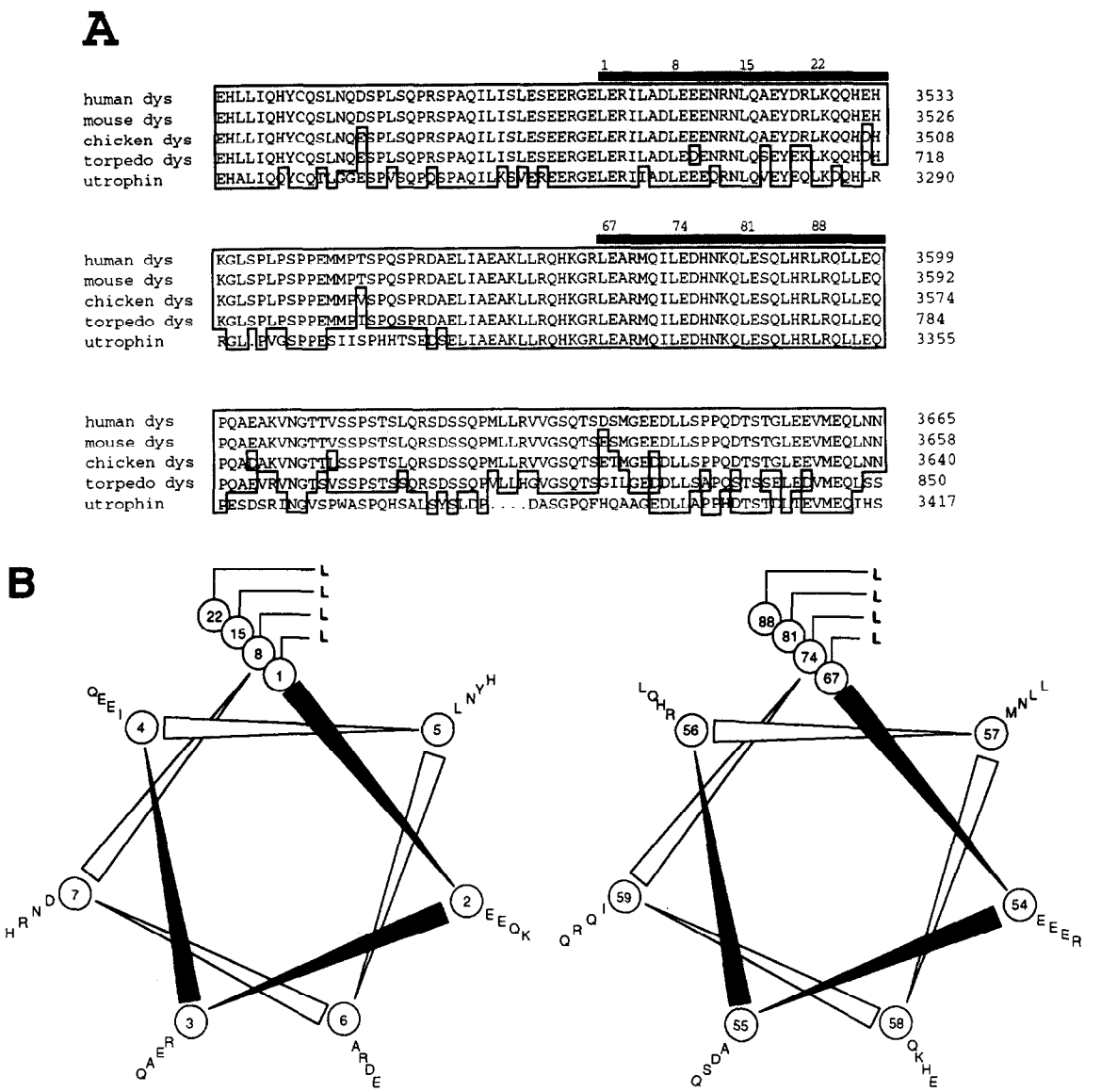


Fig. 1. (A) Amino-acid sequence comparison of the leucine zipper regions of human (aa: 3,468–3,665), mouse (aa: 3,461–3,658), chicken (aa: 3,443–3,640), and torpedo dystrophin (aa: 653–850) and the human dystrophin related protein utrophin (aa: 3,225–3,417). Sequences were aligned with PILEUP (UWGCG). Boxed regions indicate identical residues. The leucine zippers are delineated by a dark bar above the sequence. The numbers over the leucine residues in the zippers correspond to the ridge of leucines depicted in the helical wheel analysis. (B) Helical wheel analysis of the two leucine zippers of mouse dystrophin demonstrating the heptad repeat of leucines and the capacity to form a helix. Leucine 1 corresponds to residue 3,499 of mouse dystrophin.

## 3. Results

The two-hybrid system is based on the modular nature of the yeast GAL4 transcription factor. GAL4 consists of two functional domains, a DNA binding domain in the amino terminus and a transactivating domain in the carboxy terminus. When physically separated by co-expressing recombinant DNA binding domain and recombinant activating domain, the GAL4 domains no longer function as a transcription factor. However, if each domain is fused to a complementary member of a pair of interacting proteins, the two functional domains of GAL4 are brought in physical proximity and transcriptional activity can be restored. In this case, the first of the two hybrids, pGAL-Dys$H_4L_aL_b$ was generated by fusing the GAL4 DNA binding domain to a mouse dystrophin cDNA fragment encoding the fourth hinge region [15] to the second leucine zipper (amino acid residues 2,925–3,498). The second hybrid was a mouse muscle cDNA library cloned in λACT [16] such that the GAL4 activation domain was fused to each cDNA. pGAL-Dys$H_4L_aL_b$ and the cDNA library were co-transformed into y151, a yeast reporter strain carrying GAL1-*lacZ*. Two hundred thousand yeast transformants were assayed for β-galactosidase activity [16,17], and eight blue colonies were isolated. Activation domain fusion constructs were recovered from these colonies and retransformed into y151 carrying pGAL-Dys$H_4L_aL_b$ or a battery of unrelated cDNAs fused to the GAL4 DNA binding domain. All exhibited β-galactosidase activity only in the presence of the dystrophin construct, and all contained the same

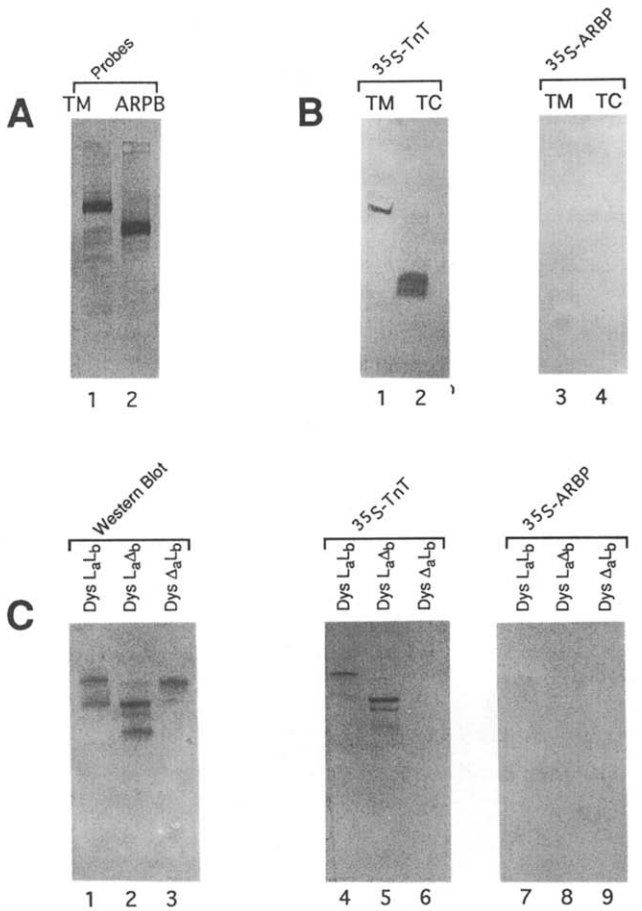


Fig. 2. (A) In vitro translation products used as probes in the blot overlay experiments (parts B and C). Lane 1: troponin T; lane 2: ankyrin repeat binding protein (ARBP). (B) Binding of radiolabelled troponin T and ARBP to known troponin interacting proteins. 2 μg of tropomyosin (TM, lanes 1 and 3) and the troponin complex (TC, lanes 2 and 4) were fractionated by 10% SDS-PAGE, immobilized on nitrocellullose, and incubated overnight with a 1:50 dilution of the probes shown in part A. Lanes 1 and 2 were probed with fast troponin T; lanes 3 and 4 with ARBP. (C) Binding of radiolabelled troponin T and ARBP to recombinant dystrophin fragments. Dys$L_aL_b$ contains both leucine zippers (aa: 3,354–3,678), Dys$L_a\Delta_b$ contains the first zipper (aa: 3,354–3,528) and Dys$\Delta_aL_b$ contains the second zipper (aa: 3,529–3,678). Lanes 1–3 were probed with anti-gene 10 antibody; lanes 4–6 with $^{35}$S-labelled troponin T; and lanes 7–9 with $^{35}$S-labelled ARBP.

gene as determined by Southern blotting and restriction mapping. They did not transactivate when genes encoding SNF1, cyclin D, p53, *rev*, *tat*, lamin, or a recently isolated ankyrin-repeat binding protein (ARBP) (P.A.P., in preparation) were substituted for dystrophin or when vector alone was co-transformed (Table 1). The lamin control is particularly significant as lamin dimerizes via a coiled-coil interaction similar to that expected of leucine zippers [18]. One of these clones was completely sequenced and was 99.2% identical on the amino acid level to *Rattus norvegicus* troponin T class IVa alpha.

To further define this interaction, several hybrid GAL4 DNA binding domain-dystrophin mutants were constructed (Table 1). Transactivation of *lacZ* by troponin T was unaffected by deletion of the second zipper or proline-rich spacer, but abolished by deletion of both zippers. Further, point mutations in the first zipper that alter the leucine of the third heptad repeat and should disrupt α-helix formation (L15P) or are expected to interfere sterically with leucine zipper mediated dimerization (L15I) also eliminated transactivation (Fig. 1 and Table 1). Therefore, troponin T interacts with the first leucine zipper in a manner consistent with the formation of a coiled coil.

As further confirmation of these interactions, we employed a physical assay based on Western blotting, similar to that used previously to demonstrate the association of an SH3 domain binding protein, 3BP-1, with c-*abl* [19] and to study the assembly of the *Drosophila Shaker* potassium channel [20]. To test the applicability of the assay, in vitro translated troponin T and ankyrin repeat binding protein control were used as probes for the troponin complex or tropomyosin immobilized on nitrocellulose (Fig. 2A). Radiolabelled troponin T but not the control probe specifically detected tropomyosin and troponin I and C (Fig. 2B). When applied to various recombinant dystrophin protein fragments containing one or both zippers, $^{35}$S-labelled troponin T interacted only when the first leucine zipper was present (Fig. 2C, lanes 4 and 5). No signal was detected from the second leucine zipper alone or from the negative control probe (Fig. 2C, lanes 6–9).

## 4. Discussion

We have presented evidence from two independent methods that a leucine zipper mediates the interaction between the carboxy terminus of dystrophin and troponin T. These data suggest a role in coupling myofilament contraction to the sarcolemma, a hypothesis advanced previously based on the observation that dystrophin co-localized with the I bands in high resolution images of longitudinal muscle sections [21–23]. Consistent with this hypothesis, the most profound defects in muscle lacking dystrophin are observed in fibers under a heavy contractile load. Type IIb fast-twitch fibers, which respond to high frequency neuronal stimulation, are preferentially affected in DMD, degenerating and regenerating before defects in the slow fibers are evident [24]. Moreover, very severe pathological changes have been observed in *mdx* mouse diaphragm relative to limb muscle and have been attributed to its higher contractile workload [25]. The uncoupling of a dystrophin mediated link between the contractile apparatus and the membrane may be responsible for the pathological response of the muscle.

In addition to coupling dystrophin to the contractile apparatus, troponin T may play a role in anchoring dystrophin to the cytoskeleton. Indeed, deletion of the actin-binding animo terminus of dystrophin in vivo results in a paradoxically mild phenotype [26]. Troponin T may be providing an alternative link between dystrophin and microfilaments.

To our knowledge, the leucine zipper-mediated interaction between two cytoskeletal proteins is novel. While functional leucine zippers have been extremely well characterized in nuclear proteins and are important for the formation of various transcription factor complexes [1], little importance has been attributed to their function in cytoskeletal proteins. The troponin T-dystrophin interaction suggests that functional leucine zippers are more widespread in the cell than previously thought.

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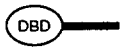
Table 1
Transactivation of β-galactosidase by various dystrophin fragments and troponin T in the two-hybrid system.

| pGAL CONSTRUCT | STRUCTURE | βACT FUSION | COLOR | β-GALACTOSIDASE |
|---|---|---|---|---|
| Dys $H_4L_aL_b$ |  | Troponin T | Blue | 137 |
| Dys $H_4L_a\Delta_b$ |  | Troponin T | Blue | 47 |
| Dys $H_4\Delta_a\Delta_b$ |  | Troponin T | White | <1 |
| Dys $H_4L_{Ile}\Delta_b$ |  | Troponin T | White | <1 |
| Dys $H_4L_{Pro}\Delta_b$ |  | Troponin T | White | <1 |
| Dys $H_4L_aL_b$ |  | ARBP | White | <1 |
| Dys $H_4L_aL_b$ |  | Vector only | White | <1 |

All dystrophin constructs were co-transformed into y151 with the GAL4 activating domain fusion indicated. Construct names indicate the extents of the dystrophin cDNA inserted ($H_4$-hinge IV, $L_a$-first leucine zipper, $L_b$-second zipper, Δ-deletion, $L_{Ile}$-isoleucine substitution in the first leucine (residue 3513) of the third heptad repeat, $L_{Pro}$-proline substitution of leucine at position 3513). Constructs have the following extents: Dys$H_4L_aL_b$ (aa: 2925-3586); Dys$H_4L_a\Delta_b$ (aa: 2925-3520); Dys$H_4\Delta_a\Delta_b$ (aa: 2925-3498); Dys$H_4L_{Ile}\Delta_b$ (aa: 2925-3520); Dys$H_4L_{Pro}\Delta_b$ (aa: 2925-3520).

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