DYC-1, a Protein Functionally Linked to Dystrophin in *Caenorhabditis elegans* Is Associated with the Dense Body, Where It Interacts with the Muscle LIM Domain Protein ZYX-1

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In *Caenorhabditis elegans*, mutations of the dystrophin homologue, *dys-1*, produce a peculiar behavioral phenotype (hyperactivity and a tendency to hypercontract). In a sensitized genetic background, *dys-1* mutations also lead to muscle necrosis. The *dyc-1* gene was previously identified in a genetic screen because its mutation leads to the same phenotype as *dys-1*, suggesting that the two genes are functionally linked. Here, we report the detailed characterization of the *dyc-1* gene. *dyc-1* encodes two isoforms, which are expressed in neurons and muscles. Isoform-specific RNAi experiments show that the absence of the muscle isoform, and not that of the neuronal isoform, is responsible for the *dyc-1* mutant phenotype. In the sarcomere, the DYC-1 protein is localized at the edges of the dense body, the nematode muscle adhesion structure where actin filaments are anchored and linked to the sarcolemma. In yeast two-hybrid assays, DYC-1 interacts with ZYX-1, the homologue of the vertebrate focal adhesion LIM domain protein zyxin. ZYX-1 localizes at dense bodies and M-lines as well as in the nucleus of *C. elegans* striated muscles. The DYC-1 protein possesses a highly conserved 19 amino acid sequence, which is involved in the interaction with ZYX-1 and which is sufficient for addressing DYC-1 to the dense body. Altogether our findings indicate that DYC-1 may be involved in dense body function and stability. This, taken together with the functional link between the *C. elegans* DYC-1 and DYS-1 proteins, furthermore suggests a requirement of dystrophin function at this structure. As the dense body shares functional similarity with both the vertebrate Z-disk and the costamere, we therefore postulate that disruption of muscle cell adhesion structures might be the primary event of muscle degeneration occurring in the absence of dystrophin, in *C. elegans* as well as vertebrates.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is due to mutations in the dystrophin gene. This gene encodes a 3685-amino acid protein, which is localized under the sarcolemma of skeletal and cardiac muscles (Koenig *et al.*, 1987; Ahn and Kunkel, 1993). In skeletal muscle, dystrophin is mainly localized at a rib-like lattice called the costamere, which is a specific muscle adhesion complex linking the Z-disk to the sarcolemma (Porter *et al.*, 1992). Dystrophin is not required for costameric organization, because costameres can form in the dystrophin-deficient *mdx* mouse (Porter *et al.*, 1992). However, the localization of costameric proteins such as β -spectrin and costameric actin tends to be affected in *mdx* mice (Williams and Bloch, 1999; Rybakova *et al.*, 2000). The mechanisms that slowly lead to muscle necrosis in the absence of dystrophin are not yet fully understood. Dystrophin clearly has a structural function, but there is still no definite proof as to

whether the pathology observed in DMD patients is due to a mechanical weakness of the sarcolemma or if it results from a secondary impairment of proteins displaced because of the absence of dystrophin (Dubowitz, 2000).

The nematode *Caenorhabditis elegans* has a dystrophin-like gene named *dys-1*, which shows extensive sequence similarities with its mammalian counterparts, including several similar key motifs. The DYS-1 protein is expressed in striated body-wall muscles where its localization overlaps actin containing thin filaments (Bessou *et al.*, 1998 and our unpublished results). Body-wall muscles are striated muscles required for the locomotion of the nematode and exhibit a sarcomeric organization similar to that of vertebrate skeletal muscles (Waterston, 1988). As in vertebrates, each sarcomere is composed of myosin containing thick filaments associated with the M-line and actin containing thin filaments anchored to a specialized adhesion structure, called the dense body (Moerman and Williams, 2006).

dys-1 mutants develop a peculiar phenotype consisting of hyperactivity, exaggerated head bending and a tendency to hypercontract (Bessou *et al.*, 1998). These mutants undergo only slight muscle degeneration (Bessou *et al.*, 1998; Grisoni *et al.*, 2003). However, in a sensitized *hlh-1(cc561)* background, which is a mild mutation of the *C. elegans* homologue of the myogenic factor *MyoD*, *dys-1* mutations lead to

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an extensive time-dependent muscle degeneration (Gieseler *et al.*, 2000).

Interestingly other genes have been identified whose mutations produce a behavioral phenotype identical to that of *dys-1* mutants: *dyc-1*, *dyb-1* (dystrobrevin), *stn-1* (syntrophin), *dgn-1* (dystroglycan), *sgn-1* (sarcoglycan), *snf-6* (an acetylcholine transporter) and *slo-1* (a potassium channel) (Gieseler *et al.*, 2000, 2001; Grisoni *et al.*, 2002b, 2003; Kim *et al.*, 2004; Carre-Pierrat *et al.*, 2006). The similarity between the phenotypes suggests that these genes may participate in the same biological function as the *C. elegans dys-1* and makes their investigation an important step in understanding dystrophin function in the nematode.

This study deals with the *dyc-1* gene, which is of particular interest because its overexpression partially compensates for the absence of dystrophin in *C. elegans dys-1*; *hlh-1* double mutants (Gieseler *et al.*, 2000). The *DYC-1* protein does not appear to have any particular motif that might help to understand its function. Its closest vertebrate relative is the CAPON protein, a putative adaptor of the neuronal nitric oxide synthase (nNOS), which is expressed in neurons and in muscle satellite cells (Jaffrey *et al.*, 1998; Segalat *et al.*, 2005).

We show that the *dyc-1* gene encodes neuronal and muscle proteins. The *dyc-1* mutant phenotypes are thoroughly characterized and shown to be due to the inactivation of the muscle isoform. We further demonstrate that mutations of *dyc-1*, like that of *dys-1*, lead to time-dependent muscle degeneration when introduced in the sensitized *hlh-1(cc561)* mutant background.

The muscle isoform of *DYC-1* was found to be associated with the dense body, which in *C. elegans* is an integrin-based muscle adhesion structure functionally related to both vertebrate Z-disks and costameres (reviewed in Lecroisey *et al.*, 2007).

The muscular *DYC-1* protein physically interacts with *ZYX-1*, the counterpart of the vertebrate focal adhesion protein zyxin (Smith *et al.*, 2002). *ZYX-1* as well as zyxin are LIM-only proteins containing three LIM domains. In vertebrates, the LIM-only protein zyxin is present at sites of cell adhesion (Crawford and Beckerle, 1991), where it interacts with α -actinin and other cytoskeletal proteins (Crawford and Beckerle, 1991), as well as with signaling proteins such as the product of the proto-oncogene *Vav* (Hobert *et al.*, 1996).

We demonstrate that the *ZYX-1* protein is expressed in striated muscles, where it localizes, like other muscle LIM domain-containing proteins of *C. elegans*, in the nucleus as well as at M-lines and dense bodies. Our results indicate that the interaction between *ZYX-1* and *DYC-1* occurs at the edges of the dense body. In addition, we show that a strongly conserved 19-amino acid stretch of the muscular *DYC-1* protein is necessary for its interaction with *ZYX-1* as well as for the localization of *DYC-1* at the dense body.

With respect to the functional relationship between *DYC-1* and the dystrophin of *C. elegans*, as a whole our results suggest that the dense body is the site of the primary events of muscle degeneration occurring in the absence of dystrophin.

MATERIALS AND METHODS

C. elegans Strains

C. elegans strains were cultured as described (Brenner, 1974). N2 Bristol strain was used as wild-type control. *MyoD/hlh-1(cc561ts)* strain (Harfe *et al.*, 1998) was obtained from B. D. Harfe (University of Florida) and A. Fire (Stanford University, Stanford, CA). *dys-1* alleles were described in Bessou *et al.* (1998).

dyc-1 alleles *cx5* and *cx32* were described in Gieseler *et al.* (2000). *zyx-1(gk190)* and *lin-15(n765)* were obtained from the *Caenorhabditis* Genetic Center (CGC). Classical genetics methods were used to construct double and triple mutants. All strains were grown at 15°C.

dyc-1:gfp and *zyx-1:gfp* Constructs and Microscopy

Reporter-gene constructs were made in green fluorescent protein (GFP)-encoding vectors (Chalfie *et al.*, 1994) pPD95.75, pPD95.77, and pPD118.20 (kindly provided by A. Fire).

To determine the expression of the *dyc-1* isoforms, we used 3 kb of genomic sequences located upstream of each isoform transcript. These regions were amplified by PCR on N2 worm DNA and cloned into the EcoRI-HindIII site of pPD95.77.

dyc-1:gfpVI is a SalI-EagI 16.3-kb genomic fragment encompassing the *dyc-1* short transcript, and containing 3.2 kb of upstream sequences, in which the *gfp* coding sequence has been inserted at the Bsu36I site (amino acid 781) of *dyc-1*.

dyc-1:gfpX is a derivative of *dyc-1:gfpVI* in which the 5' end has been extended by 6 kb by replacing the 5' most SalI-BspEI fragment by a 9.2-kb PstI-BspEI fragment.

The *dyc-1* (aa 52-81):*gfp* construct was obtained after PCR amplification of a fragment encoding amino acid (aa) 52-81 of the muscular *DYC-1* isoform. PCR was performed on cDNA clone yk259a5, (kindly provided by Y. Kohara, NIG, Japan) and the amplified fragment was cloned into the EcoRI site of pPD118.20.

The *zyx-1:gfp* plasmid was constructed by insertion into the PstI and MscI cloning sites of pPD95.75 of a 17-kb PstI-NcoI genomic fragment, which was obtained from cosmid F42G4 and corresponds to the gene F42G4.3.

The *dyc-1* (aa 52-81):*gfp* and the *zyx-1:gfp* constructs were injected in N2 wild-type animals at a concentration of 10–50 ng/ μ l along with marker pRF4 (150 ng/ μ l). All other plasmids were injected at a concentration of 1–10 ng/ μ l in *lin-15(n765)* or *lin-15(n765)*; *dyc-1(cx5)* worms with wild-type *lin-15* as a transformation marker. All injections were performed using standard procedures (Mello and Fire, 1995).

Observation of live animals under a fluorescence microscope (Zeiss Axio-plan, Le Pecq, France) was done after immobilization of the animals on a 2% agarose pad containing 0.1% sodium azide.

Production and Affinity Purification of *DYC-1-GST*

The pBRV plasmid was obtained by subcloning a 210-base pair BamHI-EcoRV fragment of the *dyc-1* cDNA (yk259a5) encoding aa 720-790, of the *DYC-1S* protein, into pGEX-3X (Pharmacia, France). To produce the *DYC-1* (aa 720-790) glutathione *S*-transferase (GST) fusion protein, *Escherichia coli* (strain BL21 DE3) transformed with pBRV were allowed to grow overnight in LB medium containing 100 μ g/ml ampicillin. Overnight cultures were diluted 1:10 in fresh medium and grown for 1 h at 37°C before adding 5 mM IPTG. Cultures were incubated for 3 h at 37°C. The bacteria were then pelleted, resuspended in 1/40 culture volume of PBS 1 $\times$, 1 mM PMSF, 1 mM iodoacetamide, 1% Triton X-100, and 1 mM EDTA and mechanically lysed by French Press (about 7000 psi in manual French Press). After centrifugation at 5500 rpm for 10 min at 4°C, the supernatant was mixed with glutathione Sepharose 4B beads (Amersham, Orsay, France) at 4°C on a rotating platform (1 ml beads for 1 l of initial *E. coli* culture). After adsorption for 1 h at 4°C, beads were collected by brief centrifugation at 1000 rpm and washed three times with 10 bead volumes of 1 $\times$ PBS. Elutions of the fusion protein were performed with 1 bead volume of 2 mM Tris, pH 8, 5 mM reduced glutathione (Sigma, Aldrich, France) and analyzed on 8% SDS-PAGE. To eliminate glutathione, purified fusion proteins were then dialyzed against 2 mM Tris, pH 8, with 12–14 MWCO membrane (Spectrum Labs, Rancho Dominguez, CA).

Production and Purification of Anti-*DYC-1* Antibodies

Polyclonal antibodies against the C-terminal end of *DYC-1* were generated (Covalab, Lyon, France) in New Zealand White rabbits immunized with ~500 μ g of *DYC-1* (aa 720-790)-GST fusion protein. The protein was injected subcutaneously, four times at 3-wk intervals.

Antibodies were affinity-purified as described by Benian *et al.* (1993). The affinity column was prepared by coupling 15 mg of purified fusion protein to 1 ml of 50/50 mixture of Affi-Gel-10 and Affi-Gel-15 (Bio-Rad, Marne la Coquette, France) according to the manufacturer's directions. The column was successively washed three times with three bead volumes of each of the following solutions: 10 mM Tris, pH 7.5; 1 mM ethanolamine, pH 8; and 10 mM Tris, pH 7.5. Five milliliters of the rabbit antiserum were prepared on DEAE Affi-Gel Blue Gel (Bio-Rad) following the manufacturer's directions. This prepared serum was precleared of anti-GST antibodies on a GST-coupled Affi-Gel 10-15 column. The cleared serum was then applied to the *DYC-1* (aa 720-790)-GST coupled column. To remove nonspecifically bound proteins the column was successively washed four times with three bead volumes of each of the following solutions: 10 mM Tris, pH 7.5; 10 mM Tris, pH 7.5, 500 mM NaCl. The bound antibodies were eluted by two bead volumes of 100 mM glycine, pH 2.5, rapidly neutralized with 2 M Tris, pH 8. The affinity-purified antibodies were concentrated on Amicon Ultra-15 (Millipore, Bedford, MA).

Immunohistochemistry

Wild-type and *dyc-1(cx32)* mutant worms were analyzed by immunohistochemistry on whole-mount preparations as in Benian *et al.* (1996). Monoclonal antibodies MH24 (anti-DEB-1/vinculin; Francis and Waterston, 1985) and affinity-purified anti-DYC-1 antibodies were used at a dilution of 1:20. Secondary goat anti-mouse (FluoroProbes 488, green, Interchim, Montluçon, France) or goat anti-rabbit antibodies (FluoroProbes 586, red, Interchim) were used at a dilution of 1:200. Images were captured with a Zeiss ZI Imager using either 63 × or 100 × oil immersion objective lenses using the Metaview software. Images were processed and annotated with Adobe Photoshop CS2 (San Jose, CA).

Immunoelectron Microscopy

Animals carrying the *dyc-1:gfpX* transgene were fixed by high-pressure freezing as previously described (Liegeois *et al.*, 2006). For immunolocalization analysis, freeze substitution was adapted from Müller-Reichert *et al.* (2003): samples were incubated in 0.01% osmium tetroxide, 0.25% uranyl acetate, and 0.25% glutaraldehyde in pure acetone for 100 h at −90°C. The temperature was then raised to −50°C at a 3°/h rate and samples left for incubation for 6 h. Samples were thoroughly rinsed with pure ethanol before resin infiltration, performed with graded concentration of Lowicryl HM20 monostep (E.M.S.). The resin was polymerized under UV light for 48 h at −50°C for 48 h at room temperature. Ultrathin sections were collected on carbon/formvar-coated nickel slot grids and processed for immunogold labeling on the Leica EM-IGL automate. The immunogold experiments were performed in PHEM buffer (60 mM PIPES, 25 mM HEPES, 20 mM EGTA, 2 mM MgCl₂, pH 6.8) as follows: three rinses in PHEM; blocking in 0.1% bovine serum albumin (Sigma), 0.1% cold water fish skin gelatin (CWFSG, Aurion, Wageningen, the Netherlands) in PHEM for 30 min; 1-h incubation in primary antibody (rabbit anti-GFP, AbCam, Cambridge, United Kingdom) diluted 1/500 in 0.1% CWFSG; six rinses in PHEM; 1-h incubation in gold-conjugated protein A (10 nm, University Medical Center, Utrecht, the Netherlands); six rinses in PHEM; post-fixation in 1% glutaraldehyde, and extensive rinsing in distilled water. Grids were then slightly contrasted in lead citrate (15 s), uranyl acetate (1 min), and lead citrate (15 s) before observation. Images were obtained with a SiS Megaview 3 CCD camera (Olympus, Münster, Germany) mounted on a FEI Morgagni transmission electron microscope (Eindhoven, the Netherlands) operated at 70 kV.

Quantification of DYC-1-GFP Immunoelectron Microscopy Labeling

Two independent scores of gold bead sarcomeric positions were performed on two different worm preparations, both of which were subjected to three different immunoreactions. We determined four different sarcomeric zones: the bottom, the middle, and the top of the dense body zone and a zone out of the dense body including the M-line. We also distinguished between gold beads localized on a dense body (in cross-sections through the dense body, when the dense body appears in black) and beads that localized between two adjacent dense bodies (at the edge), perpendicular to the cross-section plane (in cross-sections between two dense bodies, when the dense body is not visible). For the first experiment, 521 gold bead positions were analyzed in 20 worms; for the second experiment, 502 gold bead positions were analyzed in 18 worms. The results are statistically significant. Muscle cells were taken into account only when the muscle ultrastructure was correct. The means of the two different quantifications were calculated.

Quantitation of Locomotion

Locomotion was scored on active well-fed animals as previously described (Segalat *et al.*, 1995). The number of body bends/min was scored for at least 20 animals per experiment. We considered a body bend to be one complete sinusoidal movement of the scored animal.

Yeast Two-Hybrid Assay and Screening

A SacI-EcoRV cDNA fragment (cDNA clone yk259a5) encoding the DYC-1S protein (aa 9-793) was subcloned into the pAS2-1 bait vector (Clontech, Palo Alto, CA). The resulting construct, which was called pSEB, encodes a fusion protein composed of DYC-1S (aa 9-793) and the DNA-binding domain (BD) of the yeast gal4 transcription factor. Yeast cells of the CG1945 strain were transformed with pSEB.

The C-terminal end of the DYS-1 protein (aa 2857-3674) was fused to the gal4 activation domain (AD). For this purpose, a 2.4-kb *dys-1* cDNA fragment (cDNA clone yk12c11) was cloned into the polylinker of pACT2 (Clontech) with respect to the reading frame. The resulting prey plasmid was transformed into the yeast strain Y187. Interaction between DYC-1S and DYS-1 was assayed as described (Grisoni *et al.*, 2003) after mating of CG1945 yeast cells [expressing DYC-1S (aa 9-793)-BD] with Y187 yeast cells [expressing DYS-1 (aa 2857-3674)-AD]. Ten microliters of mating cultures were deposited on minimal medium lacking Leu, Trp, and His and minimal medium lacking Leu and Trp (growth control) and incubated for 3 d at 30°C.

A two-hybrid screening was performed as described (Fromont-Racine *et al.*, 1997) by mating CG1945 yeasts transformed with pSEB with Y187 yeasts transformed with a random primed *C. elegans* cDNA activation domain library (RB2) subcloned into the pACT vector (R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City). Diploid clones, 6.8 × 10⁶, containing the pSEB bait plasmid and one prey-pACT2 plasmid (from the library) were analyzed for their ability to grow on minimal medium lacking Leu, Trp, and His, after 3-d incubation at 30°C. Growth can occur only if the bait and the prey proteins interact and the *HIS3* reporter gene is transactivated. One hundred thirty-one positive clones were obtained and prey cDNAs were sequenced. Sequence analysis was performed using BLASTn searches. One hundred twenty-seven positive clones were found to correspond to the F42G4.3/*zyx-1* gene and four clones to F14F3.1.

Mapping of DYC-1- and ZYX-1-interacting Regions by Yeast Two-Hybrid Assays

A plasmid (pKG7) encoding the N-terminal region of DYC-1S (aa 9-258) fused to gal4-BD was obtained from pSEB after deletion of a SacII-PstI fragment encoding aa 259-793. The pKG7 plasmid was further used to introduce a deletion of an MboI fragment encoding aa 54-81. The resulting plasmid called pKG24 therefore encodes a fusion protein of gal4-BD and DYC-1S (aa 9-258 Δaa 54-81).

Three constructs encoding fusion proteins composed of the gal4-AD-domain and different parts of the ZYX-1 protein were obtained by subcloning the corresponding *zyx-1* cDNA fragments (cDNA clone yk247e1) into pACT2 prey vector (Clontech). Plasmid pSNXX encodes ZYX-1 (aa 68-603)-AD, pKG12 encodes ZYX-1 (aa 384-603)-AD, and pKG17 encodes ZYX-1 (aa 358-528)-AD.

Bait plasmids (pAS2-1, pSEB, pKG7, and pKG24) were transformed into the yeast strain CG1945 and prey plasmids (pACT2, pSNXX, pKG12, and pKG17) into the yeast strain Y187. Interactions between different forms of DYC-1S and ZYX-1 were assayed as described (Grisoni *et al.*, 2003) after mating of transformed CG1945 yeast cells with the transformed Y187 yeast cells. Ten microliters of mating cultures were deposited on minimal medium lacking leucine (Leu), tryptophan (Trp), and histidine (His) and minimal medium lacking Leu and Trp (growth control) and incubated for 3 d at 30°C.

GST Pulldown Assay

A SacI-SacII cDNA fragment (cDNA clone yk259a5) encoding the DYC-1S protein (aa 9-258) was subcloned into the BamHI-EcoRI cloning sites of the pGEX-2T plasmid (Pharmacia). GST and DYC-1 (aa 9-258)-GST fusion proteins were produced and purified as described above (*Production and Affinity Purification of DYC-1-GST*).

The hemagglutinin (HA)-tagged ZYX-1 (aa 15-603) protein expressing plasmid was constructed by inserting a BspEI-SnaBI *zyx-1* cDNA fragment (cDNA clone yk247e1) into the EcoRI-XhoI cloning sites of the pcDNA3.1/HA plasmid (kindly provided by J. Thomas and P. Lomonte, Lyon, France). Cos-7 cells, 10⁶, were transiently transfected with 3 μg of the ZYX-1 (aa 15-603)-HA-encoding plasmid using FuGENE 6 (Roche, Meylan, France) following the manufacturer's directions. Transfected Cos-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium (Invitrogen) containing 1% of penicillin (50 U/ml) and 1% streptomycin (50 μg/ml) supplemented with 10% fetal bovine serum.

GST pulldown assays were performed as described (Lomonte *et al.*, 2004). Cellular extracts containing the ZYX-1 (aa 15-603)-HA were prepared 48 h after transfection of Cos-7 cells with the ZYX-1-HA plasmid. Cells were washed once with 1 × PBS and centrifuged at 2000 rpm for 5 min, and the cell pellet was resuspended in 300 μl of a lysis buffer (15 mM Tris-HCl, pH 7.5, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.34 M sucrose, 0.01 mM spermidine, 0.5% Triton X-100, 0.1 M KCl, and protease inhibitors; Roche Complete mini). The suspension was incubated with shaking on ice for 45 min, and the resulting lysate was centrifuged at 13000 rpm for 10 min at 4°C. Fifty microliters of cell extracts containing ZYX-1-HA were first incubated for 1 h at 4°C with continuous mixing with glutathione-Sepharose beads coated with 50 μg GST protein to reduce the background signal. The precleared extract (50 μl) was then incubated for 2 h at room temperature with continuous mixing with glutathione-Sepharose beads coated with 50 μg GST-DYC-1 (aa 9-258) protein or GST alone as negative control. Beads were then washed three times with 1 ml of the lysis buffer. Protein complexes were eluted by boiling the beads for 5 min in 50 μl Laemmli buffer. Twenty microliters of these elutions were migrated on 8% SDS-PAGE. The presence of ZYX-1-HA protein was assayed by Western blotting as described earlier (Gieseler *et al.*, 2001) using 12CA5 anti-HA-1 primary antibody (Roche) at 1:5000 and goat anti-mouse HRP-conjugated secondary antibody at 1:3000 (Bio-Rad).

Quantification of Muscle Degeneration

For these experiments worms were grown at 15°C. Animals were fixed and stained 3 d after they reached the L4 stage. Fixation and phalloidin-rhodamine staining (Fluoroprobes, Interchim) were performed as described (Waterston *et al.*, 1984). Stained body-wall muscles were observed using a Zeiss Axioscop microscope. Only the two most visible quadrants of body-wall

Table 1. Phenotypes of *dyc-1* and *dyc-1* double mutants

Genotype	Locomotion rate (body bends/ min $\pm$ SEM)	Overbent ^a	Tendency to hypercontract
N2	14.0 $\pm$ 0.9	No	No
<i>dyc-1(cx5)</i>	18.8 $\pm$ 1.1*	Yes	Yes
<i>dyc-1(cx32)</i>	17.9 $\pm$ 1.2*	Yes	Yes
<i>dys-1(cx18);</i> <i>dyc-1(cx5)</i>	19.5 $\pm$ 1.0*	Yes	Yes
<i>dyc-1(cx5) + dyc-1:</i> <i>gfpVI</i> (1 ng/ μ l)	13.4 $\pm$ 1.2	No	No

Locomotion was scored as described in *Materials and Methods*.

^a Overbent means increased bending of the head and anterior part of the body during locomotion.

*Different from N2 ($p < 0.05$).

muscles in each animal were quantified (40 cells per animal). Twenty animals were scored for each genotype.

RNA Interference

For isoform-specific RNA interference (RNAi) experiments, an EcoRI-ScaI 350-base pair fragment encompassing exons 4–5 of *DYC-1L* and a PstI-XbaI 550-base pair fragment encompassing the first exon of *DYC-1S* were cloned into the RNAi feeding vector L4440. The resulting constructs (RS350 and PX550) were transformed into HT115 *E. coli*. RNAi was performed by feeding N2 wild-type or *DYC-1-GFPX*-expressing transgenic worms with double-strand (dsRNA)-producing *E. coli* (Timmons et al., 2001).

RESULTS

dyc-1 Mutants Exhibit *dys-1*-like Phenotypes

We identified the *dyc-1* gene in a screen for mutations producing a *dys-1*-like phenotype consisting of hyperactivity, exaggerated head bending, and a tendency to hypercontract (Gieseler et al., 2000).

The phenotypes of both *dyc-1* alleles *cx5* and *cx32* are identical and undistinguishable from that of the canonical *dys-1* allele *cx18* (Table 1). *cx5* and *cx32* were previously determined as being an early stop codon and an early frameshift, respectively (Gieseler et al., 2000). The phenotypes of *dyc-1* and *dys-1* mutations are not additive: the phenotype of *dys-1(cx18); dyc-1(cx5)* double mutants is similar to single mutants (Table 1).

Mutation of *dys-1* can lead to a progressive and severe degeneration of body-wall muscles when put in a sensitized *CeMyoD/hlh-1* background (Gieseler et al., 2000). To investigate whether the same applies to *dyc-1*, we constructed a *dyc-1(cx32); hlh-1(cc561)* double mutant and observed its muscles by performing phalloidin staining. A large number of body-wall muscle cells were found to be either missing or degenerating in this double mutant, although the phenotype was not as strong as that of the *dys-1(cx18); hlh-1(cc561)* strain (Table 2). As in the case of *dys-1*, the degenerative phenotype occurring in the *hlh-1(cc561)* background is time-dependent and affects only adults.

dyc-1 Encodes Two Isoforms with Different Expression Patterns and Functions

Analysis of EST tags shows that the *dyc-1* gene generates at least two transcripts originating from different promoters. This gives rise to two proteins with a predicted length of 793 and 887 aa that we call, respectively, *DYC-1S* (short isoform) and *DYC-1L* (long isoform; Figure 1). The sequences of the transcripts and corresponding products can be found at

Table 2. Muscle defects in *hlh-1; dyc-1* mutants

Genotype	Number of abnormal muscle cells
N2	0 $\pm$ 0
<i>dys-1(cx18)</i>	0.1 $\pm$ 0.3
<i>dyc-1(cx32)</i>	0 $\pm$ 0
<i>zyx-1(gk190)</i>	0 $\pm$ 0
<i>hlh-1(cc561)</i>	0.5 $\pm$ 0.7
<i>dys-1(cx18); hlh-1(cc561)</i>	8.8 $\pm$ 1.5***
<i>dyc-1(cx32); hlh-1(cc561)</i>	4.3 $\pm$ 1.3***
<i>zyx-1(gk190) hlh-1(cc561)</i>	0.25 $\pm$ 0.5
<i>dyc-1(cx32); zyx-1(gk190)</i>	0 $\pm$ 0
<i>dyc-1(cx32); zyx-1(gk190) hlh-1(cc561)</i>	4.65 $\pm$ 1.4***

Muscle cells were observed after phalloidin staining of animals fixed 3 d after the L4-stage. Values are mean $\pm$ SEM.

*** Different from N2 ($p < 0.001$).

www.wormbase.org. To investigate the tissue distribution of each isoform we produced GFP constructs. Approximately 3 kb of upstream sequence relative to each isoform was cloned in a GFP reporter vector and injected into wild-type animals. This experiment showed that the proximal promoter drives the expression of the gene in muscles (body-wall and vulval muscles), whereas the distal promoter drives its expression in ~ 10 of the animal's neurons (two lateral neurons that we identified to be SDQL and SDQR, plus additional nonidentified head and midbody neurons; see Figure 3; data not shown). We therefore conclude that *DYC-1S* is the muscle isoform and that *DYC-1L* is the neuronal isoform (Figure 1).

We then investigated the relationship between the phenotype of *dyc-1* mutants and the 2 isoforms. This point was addressed in two ways.

First, RNAi experiments were conducted using dsRNA specific to each of the isoforms (red bars in Figure 1). Wild-type animals were fed with bacteria producing dsRNA directed either against the muscle isoform or against the neuronal isoform. Only when the muscular isoform was inactivated did the animals display the *dys-1*-like behavioral phenotype. To verify the effectiveness of specific isoform inactivation, we repeated RNAi feeding experiments on animals carrying a *dyc-1:gfp* transgene, which is able to generate both isoforms (*dyc-1:gfpX*, Figure 1) and observed the GFP expression pattern by fluorescence microscopy (Figure 2). When these transgenic animals were fed with bacteria producing dsRNA directed against the muscle isoform, a drastic reduction of muscular GFP was observed, whereas the neuronal signal remained unaffected (Figure 2B). On the other hand, worms fed with neuronal isoform-specific RNAi exhibited a weaker but not completely absent GFP signal in neurons. In this case the muscular GFP signal seems not to be different from the control animals (Figure 2C). These observations confirm that RNAi is less effective in neurons, but also indicates that the specific inactivation of the muscular *DYC-1S* isoform is sufficient to induce the behavioral *dys-1*-like phenotype.

Second, we introduced the GFP coding sequence into a genomic fragment carrying the whole muscle isoform, but lacking the beginning of the neuronal isoform (construct *dyc-1:gfpVI*, Figure 1). The expression of this transgene is restricted to muscles thus confirming the muscle-specific expression of the short isoform. In addition, when intro-

Figure 1. Structure of the *dyc-1* gene and constructs used in this study. Top, cosmids C33G3 and C04C11 that overlap with the *dyc-1* gene. Middle, genomic organization of the *dyc-1* gene. Rectangles indicate the different exons. The *dyc-1* gene encodes two different isoforms: a short protein of 793 amino acids called DYC-1S and a long 887 amino acid protein called DYC-1L. These isoforms are generated from alternative promoters, DYC-1S is expressed in muscles and DYC-1L in neurons. Both DYC-1 proteins contain two regions of strong homology to the human protein CAPON (indicated in blue). Isoform-specific regions used in the RNAi experiments are shown as red bars. The respective positions of the *dyc-1(cx5)* and the *dyc-1(cx32)* mutations are indicated (arrows). Bottom, structure of two transgenes cited in this article. Note that the *dyc-1:gfpVI* transgene does not contain the neuronal promoter, whereas *dyc-1:gfpX* contains both the neuronal and the muscular promoter.

duced in *dyc-1* mutants, this transgene rescues the behavioral phenotype (Table 1).

Altogether, these results show that the behavioral phenotype of *dyc-1* mutants is of muscle origin. Similar findings were made previously for *dys-1*, since *dys-1* null mutants can be rescued by muscle specific *dys-1* expression (Bessou *et al.*, 1998).

Subcellular Localization of DYC-1 Isoforms in Neurons and Muscles

For observing the subcellular localization of DYC-1 in neurons and muscles, we used the *dyc-1:gfpX* construct, which is able to generate both isoforms (Figure 1).

In neurons, this reporter gene showed a striking fluorescence pattern, consisting of a string of dots running along the axon, as shown on a midbody lateral neuron SDQR in Figure 3. These structures might be attachment points for the axons to the extracellular matrix beneath them. Dots were also detected in neuron cell bodies, but it is unclear whether this corresponds to the natural localization of the protein or a transgene artifact.

In striated muscles the *dyc-1:gfpX* transgene displayed a repetitive fluorescent pattern reminiscent of the sarcomeric pattern (Figure 4). To identify which component of the sarcomere was labeled, we stained *dyc-1:gfpX*-expressing animals with the MH24 antibody, which recognizes the DEB-1/vinculin protein, one of the main components of dense bodies (Francis and Waterston, 1985; Barstead and Water-

ston, 1991). The DYC-1-GFPX protein is located at the edges of MH24 labeled dense bodies (Figure 4).

The Muscular DYC-1S Isoform Is Localized at the Edges of the Dense Body

To further investigate the subcellular localization of DYC-1 in muscles, we produced an anti-DYC-1 polyclonal antibody. After affinity purification, this antibody was used in whole-mount immunocytochemistry. Wild-type animals displayed in their body-wall muscles a spotted pattern reminiscent of the repetitive sarcomere architecture, resembling that observed in reporter gene analysis. No staining was detected in *dyc-1(cx32)* mutants, suggesting that the antibody specifically recognizes the DYC-1 protein.

We performed double-staining experiments with the anti-DYC-1 antibody and the MH24/anti-DEB-1 antibody. A detailed analysis of the pattern confirms, as observed for the DYC-1-GFPX protein, that the DYC-1 signal is located at the margins of MH24 labeled dense bodies, the *C. elegans* counterparts of vertebrate Z-disks (Figure 4). Therefore, DYC-1 seems to localize at the edges of dense bodies and might be associated with these muscle attachment structures.

Immunoelectron Microscopy Confirms Localization of DYC-1 at the Edges of the Dense Body

To analyze in more detail the dense body localization of the DYC-1 protein, we used immunoelectron microscopy.

Figure 2. Muscular and neuronal isoform-specific RNAi experiments. (A) Control animal expressing the *dyc-1:gfpX* transgene fed with bacteria containing the empty plasmid (L4440). Arrow shows the muscular expression of the transgene and arrowhead indicates the cell body of a neuron expressing the transgene. (B) Transgenic animals fed with muscular isoform specific RNAi. Note the strong reduction of muscular GFP signal, whereas the neuronal signal remains intense (arrowhead). (C) Transgenic animals fed with neuronal isoform-specific RNAi. The arrowhead points to a neuron. Note that the neuronal GFP signal is slightly reduced but not completely abolished. The muscular GFP signal (arrow) seems not to differ from control animals.

Figure 3. Neuronal *dyc-1* expression seen by reporter gene. Immunofluorescence images of wild-type animals carrying a *dyc-1:gfpX* transgene. Expression is detected in ~10 neurons. A neuron cell body (SDQR) is visible in A (arrowhead). In A and B arrows indicate the punctate pattern along the axons.

The DYC-1 antibody was not efficient enough to observe a DYC-1 signal by this approach, probably due to the low-level expression of the DYC-1 protein in wild-type worms. Therefore, DYC-1 detection was performed using a GFP antibody on *dyc-1:gfpX* transgenic worms. In the muscle cells

of these animals we observed a strong cytoplasmic background signal, probably due to the overexpression of the transgene, as well as a signal in the sarcomeric zone. We focused on gold beads located in the contractile filaments zone of the sarcomere. Clusters of gold beads or single gold

Figure 4. Subcellular localization of DYC-1 protein in muscle cells. Immunofluorescence images of wild-type animals (A–F) and *dyc-1(cx32)* mutants (G–I). (A, D, and G) Dense body localization of the DEB-1/vinculin protein labeled with MH24 antibody (red). (B) Localization of the *dyc-1:gfpX* reporter gene (green, the signal is amplified with anti-GFP antibodies). (C) Merge of A and B. In the magnification of two dense bodies, the green GFP signal is seen at the edges of the red MH24 signal. (E) Localization of the DYC-1 protein labeled with purified anti-DYC-1 antibodies (green). (F) Merge of D and E with a magnification of two dense bodies. Note that as in C, the green (DYC-1) signal is located at the edges of dense bodies (red MH24 signal). (H) *dyc-1(cx32)* mutants stained with anti-DYC-1 antibodies. No specific signal can be detected. (I) Merge of G and H with a detail of two dense bodies showing the absence of DYC-1 staining.

Figure 5. Sarcomeric localization of the DYX-1 protein in immunoelectron microscopy. (A and B) Examples of cross-sections of *dyc-1:gfpX* expressing transgenic worms labeled with an anti-GFP antibody coupled to gold beads. Note that gold beads are preferentially located at the bottom of the dense body (arrows). Clusters of gold beads in the cytoplasm (arrowheads in A), are due to overexpression of the transgene. (C) Diagram summarizing quantification of gold beads according to their sarcomeric position. These results are the mean of two different counts. 72.34% of gold beads are located in the dense body region and 27.66% outside of it. Among the gold beads which are located in the dense body zone, 4.30% are located in the dense body (3.52% at the bottom and 0.78% on the middle) and 68.04% are located at the edges (between two dense bodies), with 43.70% of them at the bottom zone, 12.02% in the middle zone, and 12.32% in the top zone. (D) Detail of a dense body showing the limits of different zones considered for scoring gold bead locations. In this picture the section does not cross the dense body (otherwise it would appear in black). Gold beads located at this level are therefore classified as being at the edge of the dense body.

beads were frequently observed at the bottom of the dense body (Figure 5, A and B). These observations indicate that DYX-1 is located in the dense body zone and primarily at the bottom of the dense body. For improved reliability, two independent scores of gold beads were performed with respect to the sarcomeric position. The results of the scores are presented in Figure 5C, and the different sarcomeric zones that were considered in these scores are indicated in Figure 5D. 72.34% of the gold beads were located in the dense body zone and only 27.66% outside this zone. In addition, gold beads were mainly located in the bottom zone of the dense body (Figure 5).

We furthermore verified whether gold beads were in the dense body or at its edges. For this purpose we compared gold-bead scoring of cross-sections that went through the dense body (in this case the dense body appeared in black) with cross-sections passing between two adjacent dense bodies. We found that only 4.30% of gold beads were located in the dense body (3.52% on the base of the dense body and 0.78% in the middle), whereas 68.04% of gold beads were located between two dense bodies (43.70% in the base zone, 12.02% in the middle zone, and 12.32% in the top zone).

Taking into account these results and the localization of the DYX-1 antibody signaling at the margin of the MH24 antibody signal (Figure 4), we conclude that DYX-1 is mainly located between two dense bodies, meaning at the edges of the dense bodies with respect to the axis of contractile filaments, and more precisely, at the bottom of these muscle attachment structures.

DYX-1 Binds to the Zyxin-like LIM Protein ZYX-1

Yeast two-hybrid experiments were carried out in order to identify potential molecular partners of the muscular DYX-1S protein.

First, we verified in a yeast two-hybrid assay whether DYX-1S interacts directly with the DYS-1 protein. To this end the DYX-1S protein (aa 9-793) was assayed for its interaction with the DYS-1 C-terminal region (aa 2857-3674). This region of DYS-1 was chosen because it contains numerous motifs potentially implicated in protein-protein interactions (Bessou *et al.*, 1998) and has been shown to mediate DYS-1 interaction with DYB-1 (dystrobrevin) and STN-1 (syntrophin; Gieseler *et al.*, 1999; Grisoni *et al.*, 2002a). However, we detected no interaction between the DYS-1 C-terminal region and the DYX-1S protein (data not shown).

Second, we used the DYX-1S protein (aa 9-793) as bait in a yeast two-hybrid screen of a *C. elegans* cDNA library. Most positive clones were found to encode the LIM domain protein ZYX-1, the counterpart of vertebrate zyxin (Smith *et al.*, 2002). In vertebrate, the LIM domain protein zyxin is present at sites of cell adhesion (Crawford and Beckerle, 1991).

ZYX-1 and DYX-1 Interact in the Muscle at the Edges of the Dense Body

To verify whether the cellular localization of the ZYX-1 protein is compatible with a physical interaction with DYX-1S, we generated worms expressing a *zyx-1:gfp* transgene. In *C. elegans*, the *zyx-1* gene is expressed in muscles and neurons (Figure 6). In muscle cells, the ZYX-1 protein is localized in the nucleus as well as at the dense bodies and M-lines

Figure 6. Subcellular localization of the ZYX-1 protein by reporter gene analysis. (A–H) Transgenic animals expressing a *zyx-1:gfp* construct. Body-wall muscles of animals labeled with anti-DEB-1 MH24 antibody (red in A and C) or anti-DYC-1 antibodies (red in D and F). (B and E) The muscle localization of the ZYX-1-GFP protein (green, the GFP signal was amplified with an anti-GFP antibody). ZYX-1-GFP localizes on dense bodies (arrowhead in B) and M-lines (arrow in E). (C and F) Merged images of A and B and of D and E, respectively, with details of two dense bodies. Note in C that the DEB-1 signal colocalizes with the ZYX-1-GFP signal on the dense body and in F that the DYC-1 signal is located at the edges of the ZYX-1-GFP labeling. (G) Localization of ZYX-1-GFP in the nucleus (arrowhead) of a body wall muscle cell. (H) Localization of ZYX-1-GFP in neurons (arrowheads).

(Figure 6). This type of localization has been reported for other muscle LIM domain proteins in *C. elegans* (reviewed in Lecroisey *et al.*, 2007).

Furthermore, we determined the localization of the ZYX-1-GFP protein at the dense body with respect to the DYC-1S protein by staining *zyx-1:gfp* transgenic worms with MH24 and anti-DYC-1 antibodies. We observed an overlapping localization of ZYX-1-GFP and DEB-1, whereas DYC-1 was located at the margins of the ZYX-1-GFP dense body dots (Figure 6).

Therefore ZYX-1 seems to be part of the dense body and interacts with DYC-1 at the edges of these muscle adhesion structures.

To investigate the function of the ZYX-1 protein and its potential functional relationship with the DYC-1S protein, we used the *zyx-1(gk190)* mutant. This mutant carries a 777-base pair deletion leading to a (predicted) ZYX-1 protein lacking all of its three LIM domains.

zyx-1(gk190) mutants exhibit no obvious behavioral phenotype. When observed after phalloidin-staining of actin

filaments the striated body-wall muscles resemble those of wild-type controls (Table 2). In the same way, no significant muscle defects could be detected in *zyx-1(gk190) hhl-1(cc561)* or *zyx-1(gk190); dyc-1(cx32)* double mutants. Furthermore, the *zyx-1(gk190)* mutation neither enhances nor reduces the muscle degeneration phenotype when it is introduced in a *dyc-1(cx32), hhl-1(cc561)* mutant background (Table 2). Together these results are consistent with observations described before, showing no drastic mutant phenotype for other *zyx-1* mutants or RNAi-mediated *zyx-1* inactivation (Smith *et al.*, 2002). This suggests that in the absence of ZYX-1, another LIM domain-containing protein might carry out the function of ZYX-1.

The Interaction between DYC-1 and ZYX-1 Involves Conserved Domains

To confirm the physical interaction between the DYC-1S and the ZYX-1 proteins, we produced a ZYX-1 (aa 15-603)-HA-tagged protein in mammalian COS-7 cells and used cell extracts for in vitro GST pulldown experiments with DYC-

Figure 7. DYC-1S interacts with ZYX-1 through its highly conserved 19 aa region. (A) Summary of results obtained with yeast two-hybrid assay. The different parts of DYC-1 and ZYX-1 proteins used in these assays are schematically indicated (top left). The table indicates the different plasmids used in these assays and the corresponding DYC-1 bait and ZYX-1 prey proteins. Pictures show plates inoculated with diploid yeasts expressing the different DYC-1S bait and ZYX-1 prey proteins. Medium without Leu and Trp serves as a positive control showing the presence of bait and prey plasmids in the tested diploid yeasts. On medium without His, Leu, and Trp, growth can occur only if the bait and the prey proteins interact. Note that negative control yeasts carrying either empty pACT2 or pAS2-1 plasmids do not grow on medium without His, Leu, and Trp. Interactions occur between the bait proteins DYC-1S (aa 9-793) and DYC-1S (aa 9-258) and the prey proteins ZYX-1 (aa 68-603) and ZYX-1 (aa 384-603; growth on medium without His, Leu, and Trp). Note that interaction is detected neither for the bait protein DYC-1S (aa 9-258 Δ aa 54-81) carrying a deletion of aa 54-81, nor for the prey protein ZYX-1 (aa 384-528) carrying only the two first LIM domains of the ZYX-1 protein (no growth on medium without His, Leu, and Trp). (B) Western blotting of GST pull-down assays. HA-tagged ZYX-1 protein (aa 15-603) was detected using an anti-HA antibody after GST pulldown performed with the GST-tagged N-terminal end of DYC-1 protein (aa 9-258) but not with GST alone. The right lane shows ZYX-1-HA expression in the total COS-7 cell extract. A volume of 20 μ l was analyzed on each lane. (C) Alignment of a DYC-1S conserved sequence. Ce, *Caenorhabditis elegans*; Dm, *Drosophila*; Ag, *Anopheles*; Rn, *Rat*; Hs, *humans*. Note that the region of 19 aa (aa 58-76 of the DYC-1S protein), which is strictly conserved from *Drosophila* to humans (two conservative differences exist in *C. elegans*, red arrows).

1-GST fusion proteins. We were thus able to show that the N-terminal aa 9-258 of DYC-1S are involved in, and sufficient for, DYC-1 interaction with ZYX-1 (Figure 7B).

To determine in more detail the protein regions required for the interaction between DYC-1S and ZYX-1, we further assayed different truncated forms of both proteins for their ability to interact, using yeast two-hybrid assays.

The ZYX-1 protein possesses three LIM domains in its C-terminal half (Smith *et al.*, 2002). We tested whether ZYX-1 LIM domains are involved in the interaction of ZYX-1 with DYC-1S. When using a portion of ZYX-1 (aa 384-603) containing all three LIM domains, interaction with DYC-1S occurs (Figure 7). However, when the third LIM domain of ZYX-1 was deleted, the remaining protein composed of aa 384-528 of ZYX-1 was no longer able to interact with DYC-1S (Figure 7). These observations indicate that the third LIM domain of the ZYX-1 protein is necessary for its interaction with DYC-1S.

Concerning the DYC-1S protein, we were able to confirm (as shown by GST pulldown assays) the importance of the N-terminal region (aa 9-258) of DYC-1S for its interaction with ZYX-1. This region interacts in yeast two-hybrid assays with ZYX-1 (aa 68-603) as well as ZYX-1 (aa 384-603; Figure 7). Interestingly, this DYC-1 sequence contains one of the two previously identified regions of similarity to the vertebrate CAPON protein (Gieseler *et al.*, 2000 and Figure 1). This sequence is a stretch of 19 aa (aa 58-76 of DYC-1S), which evolutionary has been highly conserved from worms to humans (a difference of only two residues, Figure 7C). Because a search for variants of this sequence in protein databases did not yield any significant results, it seems

likely that this motif is unique and has undergone very strong selection pressure.

To verify if this conserved region is involved in DYC-1S interaction with ZYX-1, we tested the N-terminal region of the DYC-1S protein (aa 9-258) in which aa 54-81 were deleted. In yeast two-hybrid assays, this DYC-1S (aa 9-258 Δ 54-81) protein is no longer able to interact with ZYX-1 neither with ZYX-1 (aa 384-603) nor with ZYX-1 (aa 68-603; Figure 7). This observation indicates that the conserved motif of the DYC-1S protein is required for the interaction with ZYX-1.

A Conserved Motif Addresses the DYC-1 Proteins to the Dense Body

We furthermore analyzed whether the evolutionary conserved motif of the DYC-1 protein was also implicated in the subcellular localization of the DYC-1 protein. We produced transgenic worms expressing a fusion protein composed of GFP and DYC-1S aa 52-81 under the control of a muscular promoter (the promoter of the *myo-3* gene). In striated muscles of wild-type worms the DYC-1 (aa 52-81)-GFP protein shows a sarcomeric localization, which resembles that of the endogenous DYC-1 protein, albeit slightly more diffused (Figure 8). Staining of transgenic animals with the MH24 anti-DEB-1 antibody revealed that the GFP signal partially overlaps that of MH24, indicating that the DYC-1 (aa 52-81)-GFP protein localizes at the dense body (Figure 8). This observation suggests that the conserved motif serves as a signaling sequence for addressing the DYC-1 protein to the dense body.

Figure 8. Subcellular localization of GFP fused to the highly conserved stretch of the DYC-1S protein. (A) Dense body localization of DEB-1 protein labeled with MH24 antibody (red). (B) Localization of GFP (green) fused to DYC-1S (aa 54-81) containing the highly conserved stretch of 19 aa. (C) Merged image of A and B with the detail of two dense bodies. Note that the short sequence of the DYC-1S protein is sufficient to address GFP to the sarcomeric localization.

DISCUSSION

Strategy for Identifying Proteins Functionally Related to Dystrophin

Classical genetics affords the possibility for establishing the existence of functional links between genes and their products by simply studying phenotypes, without any need for pre-existing hypotheses. Using this approach to search for proteins functionally linked to dystrophin in *C. elegans* we previously identified the DYC-1 and STN-1 proteins, which are known to be partners of dystrophin in mammals (Gieseler *et al.*, 2001; Grisoni *et al.*, 2003). In the present study, using the same approach, we established that *dyc-1* mutants share many phenotypes with *dys-1* mutants. The DYC-1 protein, which is related to the CAPON adaptor protein, is therefore likely to functionally interact with dystrophin (DYS-1).

Subcellular Localization of DYC-1

The *C. elegans* dense bodies are muscle adhesion structures and the functional counterparts of the Z-disk and the costamere of vertebrate skeletal muscles, and therefore constitute the main anchoring point of sarcomeric actin. These structures have been thoroughly studied using a combination of biochemical and genetic approaches that have shed light on their composition (Waterston, 1988; Moerman and Williams, 2006). Dense bodies are themselves anchored to the muscle cell and basement membrane via specialized proteins, all of which are also known components of vertebrate focal adhesion plaques (reviewed in Cox and Hardin, 2004; Labouesse and Georges-Labouesse, 2003; Lecroisey *et al.*, 2007).

The subcellular localization of DYC-1 is interesting. In neurons, DYC-1 is localized in discrete and regularly spaced structures along the axons. A similar localization has been described before for UNC-97 (Hobert *et al.*, 1999). UNC-97 is one of the LIM domain containing proteins, which is expressed in body wall muscles, where it localizes, like DYC-1, at dense bodies. Because dense bodies are specialized cell adhesion structures, we think that the neuronal structures labeled either by DYC-1 or UNC-97 might be attachment points of the axons.

In the muscle sarcomere we provide clear evidence that DYC-1 is located at the edges of the dense body, where filaments of sarcomeric actin are anchored via vinculin, α -actinin and other proteins. The molecular mechanisms underlying the maintenance and replacement of these muscle adhesion structures are still largely unknown. However,

these attachment points must have strong mechanical resistance properties since they sustain the bulk of the load when the muscle contracts.

Putative Role of DYC-1 in the Striated Muscle

We have shown in this article that a major binding partner of DYC-1 is ZYX-1, a LIM domain protein resembling vertebrate zyxin (Smith *et al.*, 2002). Zyxin is an adaptor protein that localizes at focal adhesion structures and binds to α -actinin and other cytoskeletal proteins (Crawford *et al.*, 1992). Zyxin may also shuttle to and from the nucleus, presumably to regulate transcription (Wang and Gilmore, 2003). In *C. elegans* striated muscles the ZYX-1 protein is localized at dense bodies as well as in the nucleus. ZYX-1 shares this dual localization with at least three other muscle LIM-domain proteins: ALP-1, UNC-95, and UNC-97 (reviewed in Lecroisey *et al.*, 2007). Like its vertebrate counterpart, ZYX-1 might be implicated in shuttling between the nucleus and the dense body and contribute to the maintenance of the stability of the dense body and the contractile machinery. However, inactivation of the *zyx-1* gene, either by RNAi depletion or the *zyx-1(gk190)* deletion allele, does not lead to an obvious mutant phenotype (Smith *et al.*, 2002 and our observations), thus suggesting a functional redundancy of ZYX-1 and other muscle LIM domain proteins. This point will be a matter for future investigation.

Even if we have not yet determined the functional relationship between ZYX-1 and DYC-1, the dissection of the protein domains required for their physical interaction allowed us to identify a functionally important motif of the DYC-1 protein.

The DYC-1 vertebrate homologue, CAPON, is thought to be an adapter protein for nNOS, thus enabling it to be directed to its targets such as DexRas1 (Jaffrey *et al.*, 1998; Fang *et al.*, 2000). Although no homologue of nNOS has yet been found in *C. elegans*, DYC-1 may also work as an adapter protein in *C. elegans*. CAPON and DYC-1 share two regions of homology, which underwent tremendous selective pressure in evolution. These two regions are predicted alpha helices, one of them carrying a motif reminiscent of a leucine zipper (Gieseler *et al.*, 2000). It is therefore likely that these regions serve as contact regions for binding partners, which may have been evolutionary conserved as well.

In this article we have provided clues as to what the function of one of these regions (aa 58-76) might be. We have shown that this sequence is implicated in the interaction of DYC-1 with ZYX-1 and serves as a signal for addressing the DYC-1 protein to the dense body.

Figure 9. Working model of dystrophin-DYC-1-dense body interactions. Dystrophin (in green) is known to interact with the Dystroglycan (DG)–Sarcoglycan (SG) complex (blue) by its C-terminal end. DYS-1/dystrophin interacts with DYB-1/dystrobrevin, which in turn binds STN-1/syntrophin. PAT-2/ α -integrin and PAT-3/ β -integrin (yellow) anchors the dense body to the sarcolemma and the extracellular matrix. DYC-1 (purple) is located at the edges of the dense body and interacts via its conserved 19-aa stretch (small purple bead) with the third LIM domain of ZYX-1/zyxin (the three LIM domains of the Zyx-1 protein are represented as small red beads). ZYX-1 is also located in the nucleus of muscle cells where it might mediate signaling pathways. We posit that there is a direct or an indirect interaction between dystrophin and DYC-1, linking DYS-1/dystrophin to the dense body.

The colocalization of ZYX-1 and DYC-1 at the dense body as well as their physical interaction suggest that both proteins participate in a common function involving a network of proteins present in the muscle attachment structure (Figure 8).

Functional Link between DYC-1 and Dystrophin

The question of dystrophin localization with respect to the sarcomere structure is of critical relevance to understanding the mechanisms underlying muscle necrosis in the absence of dystrophin. Although this has not been formally proven, increasing quantities of evidence suggest that dystrophin may be located at, or near, the Z-disk. In vertebrate, dystrophin is enriched in costameric region (Porter *et al.*, 1992); muscle-specific focal attachment plaques, which encompass the Z-disks (Pardo *et al.*, 1983) and bind to Z-disk proteins (Ervasti, 2003). In the body-wall muscles of *C. elegans*, the DYS-1 protein localizes in a broad region overlapping thin filaments and dense bodies (our unpublished results).

The similarity of the phenotypes between *dys-1* and *dyc-1* mutants is a strong argument in favor of a functional interaction between *dys-1* and *dyc-1*. In addition, it was previously reported that increasing the amount of DYC-1 is beneficial to dystrophin-deficient muscles (Gieseler *et al.*, 2000). DYC-1 might therefore serve as a functional link between dystrophin and the dense body. Because no interactions were detected in two-hybrid assays between DYC-1 and DYS-1, it is possible that a third component is involved. Alternatively, a direct interaction might exist that did not

show up in the two-hybrid assay or that implicates regions of the DYS-1 protein other than the C-terminal domain, the only domain to be assayed in this research.

Whatever the nature of the physical link between dystrophin and DYC-1, the working model that we propose from these results (Figure 9) is that DYS-1 and DYC-1 are physically linked, either directly or indirectly. The absence of DYS-1 may destabilize DYC-1. This decrease in DYC-1 function may in turn have a deleterious effect on the dense body, gradually leading to a breakdown of the sarcomere and therefore muscle degeneration.

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