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The DH-PH region of the giant protein UNC-89 activates RHO-1 GTPase in *C. elegans* body wall muscle

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Abstract

Mutation of the *C. elegans* gene *unc-89* results in disorganization of muscle A-bands. *unc-89* encodes a giant polypeptide (900 kDa) containing at its N-terminus, a DH followed by a PH domain, which is characteristic of guanine nucleotide exchange factor (GEF) proteins for Rho GTPases. To obtain evidence that the DH-PH region has activity toward specific Rho family small GTPases, we conducted an experiment using the yeast three hybrid system. The DH-PH region of UNC-89 has exchange activity for RHO-1 (*C. elegans* RhoA), but not for CED-10 (*C. elegans* Rac), MIG-2 (*C. elegans* RhoG), or CDC-42 (*C. elegans* Cdc42). The DH domain only also has similar activity for RHO-1. An *in vitro* binding assay demonstrates interaction between the DH-PH region of UNC-89 and each of the *C. elegans* Rho GTPases. Partial knock down of *rho-1* in *C. elegans* adults showed a pattern of disorganization of myosin thick filaments, similar to the phenotype caused by *unc-89* (*su75*), a mutant allele in which all of the isoforms containing the DH-PH region are missing. Taken together, we propose a model that the DH-PH region of UNC-89 activates RHO-1 GTPase for organization of myosin filaments in *C. elegans* muscle cells.

Keywords

C. elegans; muscle; GEF; rho; UNC-89; obscurin

Introduction

The small GTPases have two convertible forms; GDP bound inactivated and GTP bound activated¹. The two states are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The GEFs are reported to accelerate release of GDP from GTPases, resulting in binding to GTP in the cell, thus activating the GTPase². Rho family small GTPases include Rho, Cdc42 and Rac. Each of these GTPases are activated by specific and multiple GEFs containing a pair of domains, DH followed by PH³. Previously, we reported that UNC-89, a *C. elegans* homolog of mammalian obscurin, has the DH and PH region that is characteristic of GEFs for Rho type GTPases^{4,5}. Mutation of the *C. elegans* gene *unc-89* results in disorganization of muscle A-bands, and usually a lack of M-lines^{6,7}. *unc-89* encodes at least 6 major polypeptides, the largest of which is 900 kDa^{8,9}. Antibodies localize UNC-89

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proteins to the M-line^{4, 8} a structure at which thick filaments are cross-linked. Moreover, in *C. elegans* body wall muscle, all the M-lines are attached to the muscle cell membrane and overlying basement membrane¹⁰. Here we report evidence that the DH and PH region of UNC-89 can activate RHO-1 GTPase and that a loss-of-function mutant of *unc-89* and RNAi for *rho-1* show similar defects in the organization of thick filaments.

The DH-PH region of UNC-89 has exchange activity specifically toward RHO-1

To detect possible GEF activity of the DH-PH region of UNC-89, we performed the yeast exchange assay¹¹. The inactive forms of the GTPases cannot interact with their effectors, whereas the activated forms of the GTPases, exchanged by the GEF proteins, can interact with their effectors. In the yeast two hybrid system, the interaction between wild-type GTPases and their effectors cannot be detected, unless the GTPase has been activated by a specific GEF. Using these properties in the yeast two hybrid system, we conducted a GEF assay. The idea is that if an appropriate GEF protein is expressed in addition to the GTPase and effector, the GTPase will become activated, resulting in interaction with its effector (Figure 1A). To examine the activity of the DH-PH region of UNC-89, we prepared four yeast two hybrid constructs of *C. elegans* Rho type GTPases: RHO-1 (*C. elegans* RhoA), CED-10 (*C. elegans* Rac), MIG-2 (*C. elegans* RhoG), and CDC-42 (*C. elegans* Cdc42) fused to LexA DNA binding domain. We used specific effectors fused to the GAL4 activation domain: Rock for RHO-1, PAK for CDC42 and Kinectin for CED-10 and MIG-2¹¹. Without the GEF proteins, any combination of GTPase-effector did not show interaction. With the DH and PH region of UNC-89 protein, only the combination of the RHO-1 and its effector Rock showed interaction (Figure 1B, C). We also detected GEF activity using only the DH region of UNC-89 towards RHO-1 (Figure 1B, C).

The DH-PH region of UNC-89 interacts with Rho GTPases *in vitro*

To obtain biochemical evidence for the interaction of UNC-89's DH domain and RHO-1, we performed an *in vitro* binding assay. A total protein lysate from yeast expressing HA tagged UNC-89 DH-PH was incubated with beads coated with antibodies to HA, pelleted, washed, and incubated with bacterially expressed maltose binding protein (MBP), MBP-RHO-1, MBP-CED-10, MBP-MIG-2 or MBP-CDC-42. After pelleting and washing the beads, the proteins were eluted in Laemmli buffer and portions were separated on two gels, blotted and reacted with either antibodies to HA or antibodies to MBP. Reaction to anti-HA showed that HA-UNC-89 DH-PH was precipitated, as expected (Figure 2A, lower panel). Reaction to anti-MBP revealed that HA-UNC-89 DH-PH co-precipitated each of the MBP-GTPases, but not MBP alone (Figure 2A, middle panel).

Partial knock down of *rho-1* shows a similar defect in myosin organization as an *unc-89* mutant

To obtain evidence of an *in vivo* relationship between UNC-89 GEF and RHO-1 GTPase, we compared the phenotypes in muscle of a loss-of-function mutant of *unc-89* and *rho-1* RNAi knockdown. Among the many mutations available for the *unc-89* locus, *unc-89 (su75)* has been reported to display a complete lack of all UNC-89 isoforms that contain the DH-PH region⁸. We performed immunostaining of wild-type (N2) and *unc-89 (su75)* worms with anti-myosin heavy chain A (MHC A), to compare the pattern of thick filament organization (Figure 3A). In wild-type worms, MHC A staining shows straight lines parallel to the long axis of the muscle cells as reported previously¹². In *unc-89 (su75)* worms, MHC A staining reveals a disorganization of thick filaments including what appear to be abnormal aggregates of myosin or thick filaments, and V shaped crossing of A-bands rather than the straight lines observed in wild type muscle.

For the characterization of loss-of-function for *rho-1*, we utilized RNAi by the feeding method, since deletion or mutation of the *rho-1* locus is not available. It has been reported that *rho-1* (*RNAi*) causes embryonic lethality resulting in failure of cytokinesis in early cell divisions 13–15. To characterize the phenotype of *rho-1* (*RNAi*) at the adult stage, we observed thick filament organization in adult escapers of *rho-1* (*RNAi*). Because too few escapers were generated for immunostaining, we utilized a transgenic line that expresses MHC A with GFP tagged to its N-terminus, and shows this tagged myosin properly localized to the center of A-bands 16. When the transgenic line was fed bacteria harboring the empty RNAi vector, the GFP::MHC A showed the straight line pattern similar to anti-MHC A immunostaining of wild type worms (Figure 3B). In contrast, adult escapers from feeding RNAi of the *rho-1* gene showed disorganization of thick filaments, including aggregation and crossing lines, a pattern very similar to that observed in *unc-89* (*su75*) mutant animals (Figure 3B, upper right). In addition, adult escapers of *rho-1* (*RNAi*) displayed body wall muscle with gaps in localization of MHC A (Figure 3B, bottom right), and an Egl phenotype, suggesting a dysfunction of egg laying muscles (data not shown). Because the gaps in MHC A localization and Egl phenotype are not seen in *unc-89*(*su75*), it suggests that other GEFs can activate RHO-1 in muscle. For example, UNC-73, the *C. elegans* ortholog of Trio, is also expressed in muscle 17. Consistent with the idea that RHO-1 is downstream of UNC-89, RNAi knockdown of *rho-1* in *unc-89* (*su75*) animals, does not enhance the Unc-89 phenotype (i.e. aggregation and crossing of MHC A localization; Figure 3C, upper panels). However, in *unc-89*(*su75*); *rho-1*(*RNAi*) animals, we still observe the gapping phenotype found in *rho-1*(*RNAi*) alone (Figure 3C, bottom right).

UNC-89 is strictly localized at the M-lines, a structure known to be involved in cross-linking thick filaments, and, in nematode muscle, an attachment structure between myosin thick filaments and the muscle cell membrane and basement membrane. We demonstrated that the DH-PH region of UNC-89 activates RHO-1 GTPase, specifically. We also showed that loss-of-function mutants of *unc-89* and *rho-1* cause disorganization of myosin thick filaments. It has long been known that the Rho GTPases are involved in the organization of actin filaments 18. We propose that in striated muscle, UNC-89 and RHO-1 are involved in organization of myosin filaments around the M-lines through the activation of RHO-1. Although the molecular mechanism by which activation of RHO-1 at the M-line promotes assembly or organization of this part of the sarcomere is unknown, it is possible that the Rho effector, Rho kinase, is involved: In cultured rat heart cells, it has been shown that activated Rho stimulates assembly and organization of sarcomeres, and this effect is mediated by Rho kinase 19.

It has been reported that in *C. elegans* muscle cells, UIG-1 has specific exchange activity towards CDC-42, and that partial knockdown of *cdc-42* results in disorganization of actin thin filaments 20. UIG-1 is located specifically at dense bodies, the thin filament anchoring structures analogous to Z-disks. Taken together with our results, it is possible to propose a model that two different Rho GEFs localize to dense bodies and M-lines, respectively, and regulate independent Rho GTPases, RHO-1 by UNC-89 and CDC-42 by UIG-1.

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Figure 1. UNC-89 is an exchange factor for RHO-1

A: Principle of the Yeast Exchange Assay for detection of GEF activity. Wild type GTPase fused to LexA DNA binding domain (LexA) does not bind to its effector fused to GAL4 activation domain (GAL4-AD) and the reporter genes are not expressed (1). When a specific GEF is expressed, it catalyzes the nucleotide exchange by the GTPase, that becomes activated by binding to GTP (2). The active GTPase binds to its effector (3) thereby assembling a functional transcription factor that induces the expression of the reporter genes (4).

B: Filter assay showing specific RHO-1 activation by the UNC-89 exchange factor. Yeast strains (TAT7 or *Cdc24^{ts}* 12.1 in the case of CDC-42) were transformed by the classical lithium chloride method with expression plasmids for the indicated *C. elegans* wild type GTPases fused

to LexA DNA binding domain, myc tagged UNC-89 DH-PH or DH domain in pRs426MET or the empty vector and the appropriate effector for each GTPase fused to GAL4 activation domain: Rock for RHO-1, PAK for CDC-42 and Kinectin for CED-10 and MIG-2. Yeast strains, transformation and plasmid constructs were described previously¹¹. Individual yeast transformants were grown on nylon filters, frozen in liquid nitrogen for permeation and incubated at 37°C in Z buffer (100 mM sodium phosphate buffer pH 7, 10 mM KCl, 1 mM MgSO₄, 50 mM beta-mercaptoethanol) containing 20 µg/ml X-gal to reveal β-galactosidase activity. Reaction was stopped by incubating the filters in 10% acetic acid¹¹.

C: Liquid assay showing specific RHO-1 activation by UNC-89. TAT7 were transformed with expression plasmids encoding *C. elegans* RHO-1 or *C. elegans* MIG-2, their appropriate effectors and exchange factors as described in A. Three individual yeast transformants per combination were grown to mid log phase (OD₆₀₀= 0.5 to 1) in 5 ml liquid medium and β-galactosidase specific activity was measured in each sample as described¹¹. Briefly, cultures were pelleted, resuspended in 1 ml Z buffer and vortexed for 10 seconds after addition of 100 µl chloroform and 50 µl 0.1% SDS. Ortho-nitrophenyl-β-D-galactoside (ONPG) was added to 0.2 mg/ml as a substrate for β-galactosidase. When pale yellow color had developed, 0.5 ml of 1M Na₂CO₃ was added to stop the reaction. After centrifugation for 10 minutes, OD₄₂₀ of the supernatant was determined to quantify the concentration of ortho-nitrophenol. The activity of β-galactosidase was determined according to the formula: specific activity = OD₄₂₀/(OD₆₀₀ of the culture X time of reaction). Bar graph shows average and standard deviation of β-galactosidase activity in the three samples

A

HA-UNC-89 DH-PH	+	+	+	+	+
MBP	+	-	-	-	-
MBP-RHO-1	-	+	-	-	-
MBP-CED-10	-	-	+	-	-
MBP-MIG-2	-	-	-	+	-
MBP-CDC-42	-	-	-	-	+

anti-MBP **anti-HA****B** **CBB staining****Figure 2. The UNC-89 DH-PH region interacts with Rho-GTPases *in vitro***

Bacterially expressed MBP-GTPases interact with yeast expressed HA-UNC-89 DH-PH. Total protein extracts were prepared from yeast expressing HA-UNC-89, incubated with agarose beads coated with antibodies to HA, washed, and then incubated with purified, bacterially-expressed MBP, MBP-RHO-1, MBP-CED-10, MBP-MIG-2, or MBP-CDC-42, washed, the proteins eluted and portions of each sample were run on 2 gels and blotted. One blot was reacted with anti-HA to detect the presence of the yeast expressed protein, the other blot was reacted with anti-MBP to detect possible binding with MBP or MBP-GTPases (part A). Part B shows Coomassie Blue staining of MBP and MBP fusions used in this assay. MBP alone did not show interaction to HA-UNC-89 DH-PH, but MBP-GTPases showed interaction to HA-UNC-89

DH-PH. Especially, MBP-RHO-1 and MBP-MIG-2 showed slightly stronger interaction. For construction of plasmids expressing MBP-GTPases in bacteria, PCR-amplified and sequence-confirmed fragments of each GTPase were cloned into pMAL-KK-1, expressed and affinity-purified using amylose resin ²¹. Details about this type of in vitro binding assay were described previously ²².

Figure 3. Localization of myosin (MHC A) in *unc-89* and *rho-1* loss-of-function mutants
 A. Immunostaining of wild-type (N2) and *unc-89 (su75)* worms with anti-MHC A. Worms were fixed by the Nonet method²³ and immunostained by a procedure described previously²⁴. Anti-MHC A (5–6, 1/200 dilution)¹² localizes in a regular, straight line pattern in wild type, but shows a V shaped crossing pattern and possible aggregates in *unc-89(su75)*. Scale bar, 10 μ m. B. GFP images of GFP::MHC A expressed from a transgene in a wild type background (strain RW1596)¹⁶ with feeding of bacteria containing either empty RNAi vector or *rho-1* RNAi plasmid²⁵. The feeding RNAi procedure was described previously²⁶. Since *rho-1* RNAi causes 100% embryonic lethality, to attenuate the RNAi affect to obtain adult escapers, we fed worms with a mixture of two bacterial strains in a 1:1 ratio, one strain

containing the empty RNAi vector, and the other strain containing the *rho-1* RNAi plasmid. As compared to the empty vector control, *rho-1* adult escapers show disorganization of A-bands/thick filaments, with V shaped crossing, aggregates and gaps. Scale bar, 20 μ m. C. GFP images of GFP::MHC A expressed from a transgene in an *unc-89(su75)* mutant background with feeding of bacteria containing either empty RNAi vector or *rho-1* RNAi plasmid²⁵. We crossed *unc-89(su75)* males with RW1596 hermaphrodites in order to generate animals with the genotype *unc-89(su75); myo-3(st386); stEx30 [gfp::MHC A]*. These animals were used in the RNAi by feeding experiment. As shown on the left panel, in the background of *unc-89(su75)*, GFP::MHC A shows aggregation and V-shaped crossing similar to immunostaining shown in part A. As shown on the right panel, RNAi of *rho-1* in *unc-89(su75)* results in aggregation, V-shaped crossing and gaps in localization of GFP::MHC A. Scale bar, 20 μ m. All images were taken by using a Carl Zeiss LSM 510 confocal microscopy system and software.