The UNC-14 protein required for axonal elongation and guidance in *Caenorhabditis elegans* interacts with the serine/threonine kinase UNC-51

Ken-ichi Ogura,^{1,3} Megumi Shirakawa,¹ Thomas M. Barnes,² Siegfried Hekimi,² and Yasumi Ohshima^{1,4}

¹Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-81, Japan; ²Department of Biology, McGill University, Montreal H3A 1B1, PQ Canada

Certain *unc* mutants in the nematode *Caenorhabditis elegans*, such as *unc-14* and *unc-51*, show abnormal axonal elongation and axonal structures. We cloned the *unc-51* gene previously and predicted that it encodes a novel serine/threonine protein kinase. In this study, we precisely localized the activity to rescue an *unc-14* mutation. Also, we identified four cDNA clones encoded by the *unc-14* rescuing region, in screens for proteins that bind to UNC-51 using a yeast two-hybrid system. A mutation site in the cDNA was identified for each of the six *unc-14* mutants, establishing that the *unc-14* gene was cloned. The *unc-14* gene encodes a novel protein of 665 amino acids, and is coexpressed with the *unc-51* gene in the cell bodies and axons of almost all neurons including DD/VD and hermaphrodite-specific neurons. Another clone recovered in the two-hybrid screen encodes a carboxy-terminal region of UNC-51. Analysis using the yeast two-hybrid system suggested that a central region of UNC-14 bound to a carboxy-terminal region of UNC-51, and that the UNC-51 carboxy-terminal region oligomerized. In *in vitro* binding studies using recombinant fusion proteins, UNC-14 interacted with UNC-51 directly. We propose that UNC-51 protein kinase acts as an oligomer, and that UNC-14 is a regulator of UNC-51, in axonal elongation and guidance.

[Key Words: *C. elegans*; *unc-14*; *unc-51*; axonal elongation; axonal guidance; serine/threonine kinase]

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In the nematode *Caenorhabditis elegans*, >10 genes generally required for normal axonal guidance or elongation have been identified mutationally and analyzed in detail (Brenner 1974; Hedgecock et al. 1987; Desai et al. 1988; Siddiqui 1990; McIntire et al. 1992).

unc-5, *unc-6*, and *unc-40* genes are required for guiding circumferential elongation of pioneer axons and migration of mesodermal cells on the epidermis (Desai et al. 1988; Hedgecock et al. 1990; Siddiqui 1990; McIntire et al. 1992). The *unc-6* gene, required for both dorsal and ventral elongation and migration, encodes a *C. elegans* ortholog of vertebrate netrins, which is expressed in neuroglia and neurons along the ventral midline (Ishii et al. 1992; Wadsworth et al. 1996). Netrin-1 and netrin-2 have been identified as chemoattractants for developing axons (Serafini et al. 1994). Netrin-1 also functions as a chemorepellent (Colamarino and Tessier-Lavigne 1995). The function and expression patterns of Unc-6 and net-

rins are conserved phylogenetically from the nematode to vertebrates. The *unc-5* gene is required for dorsal elongation and encodes a transmembrane protein with immunoglobulin and thrombospondin type 1 domains (Leung-Hagesteijn et al. 1992). Mosaic analysis showed that the *unc-5* gene acts in migrating cells and pioneering neurons. The *unc-40* gene required for ventral elongation and partly for dorsal elongation encodes a nematode homolog of the vertebrate cell adhesion molecule DCC (deleted in colorectal cancer), and is expressed in motile cells and pioneering neurons (Chan et al. 1996). DCC is expressed in the nervous system in developing embryos (Chuong et al. 1994; Pierceall et al. 1994). It was revealed recently that DCC is a netrin receptor (Keino-Masu et al. 1996), suggesting that Unc-40 is a component of Unc-6 receptors (Chan et al. 1996).

Mutations in the genes *unc-34*, *unc-71*, and *unc-76* disrupt longitudinal elongation of axons along the nerve cords and their fasciculation. Therefore, these genes may encode components important for the interaction between axons or between growth cones and axons (McIntire et al. 1992; Bargmann 1993).

The genes *unc-14*, *unc-33*, *unc-44*, *unc-51*, and *unc-*

³Present address: Gene Network Laboratory, National Institute of Genetics, Mishima, Shizuoka 411, Japan.

⁴Corresponding author.

E-MAIL yohshscb@mbox.nc.kyushu-u.ac.jp; FAX 81-92-642-2645.

73 are required for circumferential axonal growth over lateral hypodermis, longitudinal axonal elongation along the nerve cords, and axonal fasciculation. Mutations in these genes lead to various abnormalities in axonal structures as well as in axonal elongation or guidance (Hedgecock et al. 1985; Desai et al. 1988; Siddiqui 1990; McIntire et al. 1992), suggesting that these genes may be primarily involved in the formation of basic axonal structures (McIntire et al. 1992). The *unc-33* gene encodes a novel protein (Li et al. 1992). Although the precise function of Unc-33 is still unknown, related proteins have been identified. A collapsin response mediator protein CRMP-62, required for collapsin-induced inward currents in *Xenopus laevis* oocytes, shares homology with Unc-33 (Goshima et al. 1995). The *unc-44* gene encodes a putative ankyrin-related protein (Otsuka et al. 1995).

Mutations in *unc-51* and *unc-14* genes show quite similar phenotypes; namely, they cause various abnormalities in many neurons, although those of *unc-14* mutations are weaker (Hedgecock et al. 1985; Desai et al. 1988; McIntire et al. 1992). For example, dorsal extensions along the lateral hypodermis of axons of the DD/VD motor neurons are prematurely terminated or misdirected in ~50% in *unc-51(e369)* and in ~20% in *unc-14(e157)* (McIntire et al. 1992). In our results, most of the DD/VD axons in *unc-51(e369)* were terminated prematurely during ventral extensions (Ogura et al. 1994) and ~20% of them in *unc-14(e57)* (data not shown). The axons of hermaphrodite-specific neurons (HSN) that are extended anteriorly along the ventral nerve cord are also terminated frequently in *unc-51* and *unc-14* mutants (Desai et al. 1988; McIntire et al. 1992). Furthermore, in *unc-14* and *unc-51* mutants, processes of DD/VD, HSN, and other GABA-ergic or serotonergic neurons examined contain many large, atypical varicosities. Ultrastructural examination of *unc-14* and *unc-51* mutants revealed defects in membranous structures, including significant enlargements in axon diameter and many abnormal vesicles and cisternae-like structures within axons (McIntire et al. 1992). These structural abnormalities in axons are similar to those found in the temperature-sensitive *shibire* mutant of *Drosophila* (Koenig et al. 1983; Kosaka and Ikeda 1983), as pointed out by McIntire et al. (1992). The *shibire* mutant shows blockage of neurite development and growth cone formation in neuronal cultures at the restrictive temperature, and these defects were presumed to be related to defective membrane recycling and endocytosis (Kim and Wu 1987). Thus, it is possible that the *unc-51* gene is involved in axonal membrane recycling (Ogura et al. 1994).

We cloned the *unc-51* gene previously and showed that it encoded a predicted novel serine/threonine protein kinase (Ogura et al. 1994). The kinase domain is essential for the function, because a point mutation in the predicted ATP binding region resulted in loss of rescuing activity. An *unc-51::lacZ* fusion gene was expressed in almost all neurons at all stages, and this fusion product was localized to axons and cell bodies. These results suggest that UNC-51 acts in axons and regulates axonal

elongation and fasciculation by phosphorylation. Because many phosphorylated proteins have been found in the nervous system, related kinases probably function in the nervous system of other organisms as well (Manser et al. 1994; Hill et al. 1995). To analyze the function of UNC-51 further, we searched for UNC-51 binding proteins using a yeast two-hybrid system (Fields and Song 1989; Chien et al. 1991). In this approach and by positional cloning, we identified the *unc-14* gene and we show that UNC-51 can interact with UNC-14 directly.

Results

Positional cloning of *unc-14*

unc-14 had been mapped genetically between the genes *sem-4* (Basson and Horvitz 1996) and *myo-1* (Dibb et al. 1989), which probably corresponds to *let-75* (cited in Starich et al. 1996). Using a pool of 11 overlapping cosmid clones that covered this region (Fig. 1A), we were able to rescue the locomotory defect of *unc-14(e57)*. The rescuing pool was split into three overlapping subpools, only the middle one of which was able to rescue (Fig. 1A). This indicated that the rescuing region was centered on cosmid R05H7. We generated a genomic restriction map encompassing R05H7 and flanking regions, and determined the extents of several additional clones from the physical map that lay in this region. The activity of rescuing the *unc-14* mutation was shown to lie in the 15-kb overlap between R12F2 and R01B1 (Fig. 1B). A series of subclones was tested for rescue (Fig. 1C), which showed that the 4.8-kb region between the *HindIII* boundary of pRA18 and the *XhoI* boundary of pR9.2 was sufficient to rescue the *unc-14* mutation.

Identification of proteins interacting with UNC-51 by using a yeast two-hybrid system

In an independent approach, we sought proteins interacting with UNC-51 using a modified yeast two-hybrid system (Durfee et al. 1993). About 5×10^5 clones of a *C. elegans* cDNA library were screened, and 16 positive clones were obtained and partially sequenced. One positive clone out of the 16 was an *unc-51* cDNA, but encoded only the carboxy-terminal region (Fig. 2A). Four other clones were overlapping with one another (Fig. 2B), and matched cDNA clones isolated by Y. Kohara (pers. comm.). The latter cDNA clones had been positioned by hybridization to three YAC clones, Y39A9, Y51D6, and Y53F1, around the *unc-14* locus (Fig. 1A; Y. Kohara, pers. comm.). Because *unc-14* mutants have defects quite similar to those of *unc-51* mutants, as described in the Introduction, we considered that these cDNA clones could correspond to the *unc-14* gene. To examine this possibility, we hybridized the cDNA clone to several cosmids and subclones in the *unc-14* region. Cosmid R05H7 and the three subclones indicated in Fig-

Figure 1. Localization of *unc-14* rescuing activity. (A) Selected clones from the physical map of the region are shown. The upper three lines correspond to YAC clones hybridizing to the *unc-14* cDNA, whose precise endpoints are not known. The three subpools tested are indicated by different shadings. Note that C03C3, F21E6, and C32D3 were common to two different subpools. (B) Restriction mapping and rescue data for whole cosmids centered on the *unc-14* region. Note that K06F6 was a corrupted clone, but contained the indicated region contiguously. The bracket at the bottom indicates the *unc-14* rescuing region. (C) Rescue and hybridization data with subclones. Clones reacting with the cDNA probe are indicated. The bracket at the bottom indicates the *unc-14* rescuing region. (Sc) *SacII*; (C) *Clal*; (X) *XhoI*; (E) *EcoRI*; (H) *HindIII*; (S) *SalI*. Note that the large *Clal* fragment contains two internal *Clal* sites (C*) that were probably protected by overlapping dam methylation.

ure 1C were positive, showing that the cDNA and the *unc-14* rescuing region were coincident (Fig. 1C).

The longest cDNA clone was sequenced completely. Also, PCR analysis with both trans-spliced leaders SL1 and SL2 (Huang and Hirsh 1989) showed that the mRNA begins with SL1. The size of the mRNA deduced from the cDNA is ~2.8 kb. Northern hybridization of poly(A)⁺ RNA from a population of mixed stages with a cDNA probe showed a band corresponding to this size, besides 3.5- and 1.8-kb bands probably resulting from cross-hybridization with rRNAs (data not shown). The mRNA is predicted to encode a protein of 665 amino acid residues (Fig. 3). BLAST analysis of the entire sequence of this predicted protein revealed no remarkable homology (30% or more identify for a region of 100 amino acids or more, or a significant homology with a family of pro-

teins) with the proteins predicted from the DDBJ nucleic acid sequence database. Surprisingly, the rescue data indicated that the carboxy-terminal region of UNC-14 was not required for rescue of locomotion: A fragment extending only down to the *HindIII* site (amino acids 1–370, corresponding to 56% of UNC-14) readily rescued *unc-14(e57)*. On the other hand, C44A7, which lacks the *unc-14* promoter, 5' untranslated region (UTR), and first few codons, did not rescue *unc-14(e57)*. Genomic sequence analysis revealed that the *unc-14* gene has 10 exons (Fig. 4). cDNAs from the six known *unc-14* mutants were sequenced completely, and all had nonsense mutations near the amino terminus of the protein (Figs. 3 and 4; Table 1). These results establish that this novel protein is the *unc-14* gene product.

Genetic interaction between *unc-51* and *unc-14* genes was examined by introduction of an *unc-14* rescuing clone K06F6 or pR9.2 into *unc-51(e369)* mutant, which was not rescued. Introduction of an *unc-51* rescuing clone (pF14) into *unc-14(e57)* was not successful. This result was possibly attributable to toxic overexpression of the *unc-51* gene, because rescue of the *unc-51* mutation with the *unc-51* clone was also difficult.

Expression patterns of *unc-14* and *unc-51* genes

To analyze the expression pattern of *unc-14*, we constructed an *unc-14::lacZ* fusion gene that was capable of rescuing *unc-14(e57)*. This fusion gene was expressed in almost all neurons in all stages, especially in cell bodies and axons (Fig. 5). As the expression pattern is very similar to that of an *unc-51::lacZ* fusion gene (Ogura et al. 1994), UNC-14 and UNC-51 appear to be coexpressed in most neurons.

To examine coexpression of *unc-14* and *unc-51* genes in DD/VD and HSN neurons that show axonal defects

Figure 2. The cDNA clones isolated by the two-hybrid screening. The numbers represent the positions of the 5' or 3' ends of the cDNAs in comparison with the full-length cDNA. (A) An *unc-51* cDNA clone; (B) *unc-14* cDNA clones.

Figure 4. Genomic structure of the *unc-14* gene. (ATG) A putative initiation methionine; (STOP) the stop codon; (Poly A) a putative polyadenylation signal. The mutation sites in the six *unc-14* mutants are indicated by their allele names. Boxes represent exons. Solid boxes represent the UNC-51 binding domain; shaded boxes represent the other coding regions. SL1 shows the splice leader sequence. Although we had originally sequenced this region, it was later sequenced by the *C. elegans* genome project. K10D3.2 gene predicted by the Gene Finder corresponds to the UNC-14 coding region, but has an incorrect splicing pattern, in which exon 7 is linked to exon 10.

tions, the interacting regions were analyzed by using the yeast two-hybrid system (Table 2). A central region of UNC-14 (amino acid residues 200–382) interacted with a carboxy-terminal region of UNC-51(455–856) downstream of the kinase domain. Also, this same carboxy-terminal region of UNC-51 was able to self-associate (Table 2; Fig. 7). These results suggest that UNC-51 acts as an oligomer and that UNC-14 interacts with UNC-51 in the oligomerization domain outside of the kinase domain.

Association of UNC-51 and UNC-14 in vitro

To confirm the direct interaction between UNC-51 and UNC-14, interactions of recombinant proteins produced by *Escherichia coli* were analyzed. The carboxy-terminal region of UNC-51(455–856) and a mutant form (R841H) of the same UNC-51 region, corresponding to the *e1120* mutation (Ogura et al. 1994), were expressed as fusions with glutathione *S*-transferase (GST). The GST fusion proteins were immobilized on glutathione-Sepharose 4B beads. A fragment of UNC-14(1–382) including the amino terminus and the UNC-51 binding region was expressed as a fusion with maltose binding protein (MBP). The GST–UNC-51 fusion proteins immobilized on beads were mixed with an extract of *E. coli* that produced MBP–UNC-14(1–382). The interaction among these pro-

Table 1. Mutation sites of *unc-14* alleles

Alleles	Mutation site (codon)
<i>e57</i>	75 W (TGG) → STOP (TGA)
<i>e157</i>	76 Q (CAA) → STOP (TAA)
<i>e1119</i>	86 W (TGG) → STOP (TAG)
<i>e866</i>	106 W (TGG) → STOP (TGA)
<i>e737</i>	122 S (TCA) → STOP (TAA)
<i>e608</i>	169 Q (CAA) → STOP (TAA)

Figure 5. Expression patterns of an *unc-14::lacZ* fusion gene. The *SalI* fragment of pR9.2 shown in Fig. 4 was subcloned in *lacZ* expression vector pPD22.04. This *unc-14::lacZ* fusion gene is capable of rescuing an *unc-14* mutant. The transgenic worms were fixed and stained with an anti-LacZ antibody. (A) L2 worm head region. (H.G.) head ganglia. (B) The posterior part of the same worm. (T.G.) Tail ganglia. (C) A higher magnification picture of the middle-body region of an L2 worm. White arrows represent cell bodies of ventral neurons. Black arrowheads represent the ventral nerve cord. Bar, 50 μ m.

teins was observed by Western blotting and an anti-MBP antiserum. The carboxy-terminal region of UNC-51(455–856) and the 1–382 region of UNC-14 interacted directly, and the mutation in the carboxy-terminal region of UNC-51 reduced the binding dramatically (Fig. 8). Attempts in vitro to detect self-association of the UNC-51 carboxy-terminal region were not reproducibly successful. We presume that this result was either because the produced UNC-51 fusions had already self-associated or because the self-association was not strong enough to be detected reproducibly under the conditions used.

Discussion

UNC-14 and UNC-51 can interact directly

By positional cloning, we identified the *unc-14* gene and showed that it encoded the cDNAs isolated in a two-hybrid screen with an UNC-51 bait (Figs. 1 and 2).

Figure 6. Expression of *unc-14::gfp* and *unc-51::gfp* fusion genes in DD/VD and HSN neurons. Green represents GFP fluorescence, and red (Texas Red) represents neurons stained with an anti-GABA antibody or HSN neurons stained with an anti-serotonin antibody. A Zeiss LSM410 confocal laser scanning microscope was used in these analyses. Bar, 50 μ m. (A–F) Transgenic animals that express an *unc-14::gfp* promoter fusion gene. (A,D) *unc-14::GFP* (green). (B,E) Staining with an antibody to GABA or serotonin (red). (C,F) Merge of A and B or D and E. The yellow color indicated by the arrows represents coexpression. (G–L) Transgenic animals that express an *unc-51::gfp* promoter fusion gene. (G,J) *unc-51::GFP* (green). (H,K) Staining with an antibody to GABA or serotonin (red). (I,L) Merge of G and H or J and K. Yellow color indicated by arrowheads represents coexpression. The total number of cell bodies stained by the GABA antibody, including those in other focal planes, in a whole animal is ~20. They include most of the 19 DD/VD neurons (McIntire et al. 1992), and most of them express *unc-14::GFP* and *unc-51::GFP*.

Table 2. Interaction between UNC-51 and UNC-14, and UNC-51 self-interaction in the yeast two-hybrid system

Activation domain fusion	Binding domain fusion				Snf1
	UNC-14 (1-665)	(UNC-51)			
		(1-856)	(455-856)	1-288)	
UNC-51 (1-856)	+	+	-*	-	-
UNC-51 (1-288)	-	-	-	-	N.D.
UNC-51 (289-856)	+	+	-**	N.D.	-
UNC-51 (160-655)	-	-	-	N.D.	N.D.
UNC-51 (656-856)	-	-	-	N.D.	N.D.
UNC-51 (455-856)	+	+	+	-	-
UNC-14 (1-665)	-	+	+	-	-
UNC-14 (126-643)	-	+	+	N.D.	-
UNC-14 (200-665)	N.D.	+	+	N.D.	-
UNC-14 (1-382)	-	+	+	-	-
UNC-14 (1-522)	-	+	+	-	-
UNC-14 (200-382)	-	+	+	-	-
UNC-14 (383-665)	N.D.	-	-	N.D.	N.D.
SNF4	-	-	-	N.D.	+

β -Galactosidase activities were determined by a filter assay (Durfee et al. 1993) in Y190 yeast strain containing the indicated plasmids. (+) A positive result in which at least several colonies changed to blue in 24 hr; (-) A negative result in which no colony changed to blue in 24 hr. (N.D.) Not done. Contradictory results in the sense that the result of the reverse pair of fusions as to binding and activation domains was + (*) or that the expected result should be + (**). We believe that conformation or stability of the fusion protein may be responsible for these results.

The *unc-14* gene encodes a novel protein (Fig. 3), whereas *unc-51* encoded a putative neuronal protein kinase (Ogura et al. 1994). Bacterially expressed recombinant proteins revealed direct interactions between UNC-14 and UNC-51 (Fig. 8). An UNC-14::LacZ fusion protein was localized to neuronal axons and cell bodies (Fig. 5). Its expression pattern overlapped that of an UNC-51::LacZ fusion protein (Ogura et al. 1994). In particular, both *unc-14* and *unc-51* genes are expressed in DD/VD and HSN neurons (Fig. 6). Furthermore, *unc-51* and *unc-14* mutants share similar defects in axonal outgrowth and abnormal axonal structures (see introductory section). Thus, we suggest that UNC-51 and UNC-14 interact in neurons, and this interaction is required for normal axonal elongation and/or guidance.

Figure 7. Schematic representations of the interaction between UNC-14 and UNC-51, and of the UNC-51 self-interaction. The region in UNC-14 between the dotted line (at position 370) and the carboxyl terminus is not essential for the rescue of *unc-14* mutant phenotypes in germline transformation (see Results).

Interaction between UNC-14 and UNC-51 and oligomerization of UNC-51 are important for their function

We found that the carboxy-terminal region of UNC-51 could bind to a central region of UNC-14 (Table 2; Figs.

Figure 8. In vitro binding studies. GST, GST-UNC-51(455-856) fusion, MBP, or MBP-UNC-14(1-382) fusion were expressed in *E. coli*. GST or a GST fusion was immobilized on beads and the beads were mixed with an extract including MBP or an MBP fusion. MBP or MBP fusion proteins bound to the beads were detected by Western blotting analysis using an anti-MBP antiserum as described in the Materials and Methods. (Lane 1) GST and MBP-UNC-14; (lane 2) GST-UNC-51 and MBP-UNC-14; (lane 3) GST-UNC-51 and MBP; (lane 4) GST-M.UNC-51 and MBP-UNC-14. The predicted molecular weight of the MBP-UNC-14 is 87 kD, and the multiple bands shown in lanes 2 and 4 are probably attributable to protein degradation.

7 and 8), and a mutation in the carboxy-terminal region of UNC-51 decreased the binding dramatically (Fig. 8). In addition, because each of the six *unc-14* alleles analyzed in this study has a nonsense mutation near the amino terminus of UNC-14 (Figs. 3 and 4; Table 1), the UNC-51 binding domain should not be translated in these mutants. The carboxy-terminal region of UNC-51 (455–856), which is necessary for UNC-14 binding, was shown previously to be important for the UNC-51 function by the severe phenotype of *unc-51(e369)* nonsense mutation at amino acid 553 within the carboxy-terminal region (Ogura et al. 1994). All these results suggest that the interaction between UNC-14 and UNC-51 is important for their respective functions. We have also shown that the carboxy-terminal region of UNC-51 self-associated in the yeast two-hybrid system (Table 2; Fig. 7). This suggests that oligomerization of UNC-51 kinase is also important for its function. Consistent with this, two groups reported recently that artificial oligomerization activates the serine/threonine kinase Raf-1 (Farrar et al. 1996; Luo et al. 1996).

Functions of UNC-14 and UNC-51

What is the function of UNC-14? UNC-14 is likely to be required for UNC-51 function, because UNC-14 binds to UNC-51 and because all of the *unc-14* alleles are recessive nonsense mutations and therefore probably have lost the function. We propose the following three models consistent with this notion. In the first model, UNC-14 is a regulator, possibly an activator, of UNC-51. In this model, UNC-51 exists in an inactive (and possibly oligomerized) form, and binding of UNC-14 to the carboxy-terminal region of UNC-51 activates the kinase activity as with the binding of Cdc42 to p21-(Cdc42/RAC) activated kinase (PAK) (Manser et al. 1994) or TAK1-binding protein 1 (Tab1) to transformation growth factor (TGF β)-activated kinase 1 (Tak1) (Shibuya et al. 1996), or stabilizes the active oligomer of UNC-51 (Fig. 9A). In the

second model, UNC-14 recruits the UNC-51 to a certain intracellular region (Fig. 9B), like the function of Lin-7 in relation to Let-23 (Simske et al. 1996) or that of NMY-2 to PAR-1 (Guo and Kemphues 1996). For example, UNC-51 may be brought from a cytoplasmic location to the axonal membrane by UNC-14, where it phosphorylates its substrate. This model is consistent with the finding that *unc-14* and *unc-51* mutants have a defect in axonal membrane structures (see Introduction). In the third model, UNC-14 may tether UNC-51 to another factor or substrate (Fig. 9C) as Ste5p functions in the regulation of Ste11, Ste7, and Fus3 (Choi et al. 1994). These models are not always mutually exclusive. In all of the above models, UNC-14 is not presumed to be a substrate of UNC-51 kinase. Although UNC-14 could possibly be a substrate, we prefer the models in which it is not a substrate. Our view is based on the results that the interaction between the UNC-51 kinase domain and UNC-14 was not detected in the two-hybrid system. A substrate of UNC-51 has yet to be identified.

McIntire et al. (1992) showed that *unc-14(e57)* mutant had less severe defects compared with *unc-51(e369)*, except for the circumferential elongation of HSN neurons. In addition, *unc-14(e57)* mutant worms are somewhat motile in contrast to *unc-51(e369)*. *unc-14(e57)* is predicted to produce a protein of only 74 amino acids suggesting that this allele is null. *unc-51(e369)* nonsense mutation at position 553 (Ogura et al. 1994) results in the loss of most of the carboxy-terminal UNC-14 binding region. If the function of UNC-14 is absolutely required for UNC-51, the phenotypes of the null or severe mutants in these genes should be the same. The phenotypic difference between these mutants suggests that UNC-51 partially functions without UNC-14, or that another positive regulator of UNC-51 may exist.

The reporter constructs of *unc-51* and *unc-14* genes are strongly expressed also in adults in many neurons (Fig. 6; Ogura et al. 1994), long after the time when the axons affected in the mutants are extending. Therefore, it is possible that UNC-51 and UNC-14 are required not only

for axonal elongation and guidance but also either for maintenance or for an unknown neural function in the adult.

Materials and methods

C. elegans strains

Wild-type strain N2, CB369(*e369*) and CB57(*e57*) were provided by *Caenorhabditis* Genetics Center. *unc-14* mutant strains CB157(*e157*), CB608(*e608*), CB737(*e737*), CB866(*e866*), and CB1119(*e1119*) were provided by J. Hodgkin (Medical Research Council, UK). *lin-15*(*n765*) was provided by P. Sengupta and C. Bargmann (University of California, San Francisco). Worms were grown on the NGM agar plates with OP50 strain of *E. coli* at 20°C (Brenner 1974; Wood 1988) except for *lin-15*(*n765*), which was grown at 15°C.

Molecular manipulations

Molecular manipulations of DNA or RNA were performed essentially according to Sambrook et al. (1989). The mutation sites were located by sequencing reverse transcription-PCR products of the total RNA of the mutants. The accession number in DDBJ/EMBL/GenBank for the *unc-14* cDNA sequence reported in this paper is AB000913.

Yeast two-hybrid screening and analysis

The *C. elegans* cDNA library in λ ACT vector for two-hybrid screen in this study was kindly provided by R. Barstead (Oklahoma Medical Research Foundation, Oklahoma City). Yeast strain Y190 was used as the host. Vectors used in this study were pAS2 (for fusion with GAL4 DNA-binding domain) and pACT or pACT2 (for fusion with GAL4 transcriptional activator domain). For library screening, an *unc-51* cDNA fragment corresponding to the full-length UNC-51 (amino acids 1–856) was used. Library screening and β -galactosidase filter assays were performed as described in Durfee et al. (1993).

In vitro binding

The carboxy-terminal region of wild-type UNC-51 (amino acids 455–856) and M.UNC-51 (amino acids 455–856) carrying R841H mutation corresponding to *e1120* were expressed as GST fusion proteins by using pGEX-KG vector (Guan and Dixon 1991). pMALc2 (New England Biolabs) was used for expression of MBP-UNC-14 (amino-terminal region, amino acids 1–382) fusion proteins. *E. coli* strain BL21(DE3) harboring pT-Trx was used for producing soluble fusion proteins (Yasukawa et al. 1995). Fusion proteins were induced by 1 mM IPTG at 37°C for 3 hr. The *E. coli* cells that produced recombinant proteins were sonicated on ice in 1× PBS (140 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄) containing 0.1% Triton X-100 and 10 mM β -mercaptoethanol. About 2- μ g of GST, GST-UNC-51, or GST-M.UNC-51 was immobilized on 50 μ l of glutathione-Sepharose 4B beads (Pharmacia Biotech) and purified. The beads were mixed with an extract of *E. coli* that produced MBP (~4 μ g) or MBP-UNC-14 (~1 μ g) in 1× PBS containing 0.1% Triton X-100 and 10 mM β -mercaptoethanol. The beads were washed several times in the buffer for sonication. Each sample was fractionated by 8% SDS-PAGE and analyzed by ECL Western blotting (Amersham). We used an anti-MBP antiserum (New England Biolabs) for the primary antibody, and HRP conjugated anti-rabbit IgG (Cappel) for the secondary antibody.

Rescue of an *unc-14* mutation

Following the method of Mello et al. (1991), cosmid or plasmid DNA was injected into the syncytial gonads of adult hermaphrodites of an *unc-14* mutant at a total concentration of 100 ng/ μ l or 1 ng/ μ l (Barnes et al. 1996). pPD10.46 (Fire et al. 1991) or pRF4 carrying *rol-6^d* gene (Kramer et al. 1990) was used for the cotransformation marker.

Construction and expression of lacZ fusion or gfp fusion constructs

The 5.4-kb *Sa*I fragment of the *unc-14* gene was subcloned into the *Sa*I site of the *lacZ* expression vector pPD22.04 (Fire et al. 1990). This plasmid was named p04SS. For transformation, a 1:1:8 mixture of p04SS, pR9.2 (intact *unc-14* gene), and pBlue-script SK+ (Stratagene, for dilution) at a total concentration of 100 ng/ μ l was injected into *unc-14*(*e57*) mutant. Non-Unu transgenic worms were selected. Although this *unc-14::lacZ* fusion gene had a nuclear localization signal, no nuclear staining was observed. This *unc-14::lacZ* fusion gene is capable of rescuing *unc-14*(*e57*) mutant, when injected together with pPD10.46.

For coexpression analysis, the putative promoter region of the *unc-51* gene (4-kb *Eco*RI–*Bst*XI fragment; Ogura et al. 1994) was subcloned into the *Xma*I site of the *gfp* expression vector pGFP-TT (kindly provided by Y. Jin and H.R. Horvitz, Massachusetts Institute of Technology, Cambridge). This plasmid was named pTTu51P. The putative promoter region (1.4 kb) of the *unc-14* gene was amplified by PCR and subcloned into the *Xma*I site of pGFP-TT (pTTu14P). For transformation, a 1:1 mixture of pTTu51P or pTTu14P and pJM23 carrying *lin-15*(+) gene (Huang et al. 1994) was injected into *lin-15*(*n765*) hermaphrodites at a total concentration of 100 ng/ μ l. Non-Lin worms with a normal vulva grown at 25°C were selected as transgenic animals.

Indirect immunofluorescence staining with an antibody

Staining with an anti-LacZ antibody (Cappel) was performed essentially according to the protocol described by Finney and Ruvkun (1990) except for fixation (2% paraformaldehyde and 1× MRWB for 45 min at 4°C). The specimens were observed in a Zeiss Axioplan fluorescence microscope. Simultaneous visualization of GFP and GABA or serotonin by an anti-GABA antibody (Sigma) or an anti-serotonin antibody (purchased from H. Steinbusch, University of Limburg, Maastricht, Germany) was performed by the same protocol as for anti-serotonin staining (Link et al. 1992; McIntire et al. 1992). The specimens were observed using a confocal laser scanning microscope (Zeiss, LSM410).

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References

- Bargmann, C.I. 1993. Genetic and cellular analysis of behavior in *C. elegans*. *Annu. Rev. Neurosci.* **16**: 47–71.
- Barnes, T.M., Y. Jin, H.R. Horvitz, G. Ruvkun, and S. Hekimi. 1996. The *Caenorhabditis elegans* behavioral gene *unc-24* encodes a novel bipartite protein similar to both erythrocyte band 7.2 (stomatins) and nonspecific lipid transfer protein. *J. Neurochem.* **67**: 46–57.
- Basson, M. and H.R. Horvitz. 1996. The *Caenorhabditis elegans* gene *sem-4* controls neuronal and mesodermal cell development and encodes a zinc finger protein. *Genes & Dev.* **10**: 1953–1965.
- Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.
- Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* **263**: 802–805.
- Chan, S.S.-Y., H. Zheng, M.-W. Su, R. Wilk, M.T. Killeen, E.M. Hedgecock, and J.G. Culotti. 1996. UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* **87**: 187–195.
- Chien, C.T., P.L. Bartel, R. Sternglanz, and S. Fields. 1991. The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci.* **88**: 9578–9582.
- Choi, K., B. Satterberg, D.M. Lyons, and E.A. Elion. 1994. Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. *Cell* **78**: 499–512.
- Chuong, C.-M., T.-X. Jiang, E. Yin, and R.B. Widelitz. 1994. cDCC (chicken homologue to a gene deleted in colorectal carcinoma) is an epithelial adhesion molecule expressed in basal cells and involved in epithelial-mesenchymal interaction. *Dev. Biol.* **164**: 383–397.
- Colamarino, S.A. and M. Tessier-Lavigne. 1995. The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell* **81**: 621–629.
- Desai, C., G. Garriga, S.L. McIntire, and H.R. Horvitz. 1988. A genetic pathway for the development of the *Caenorhabditis elegans* HSN motor neurons. *Nature* **336**: 638–646.
- Dibb, N.J., I.N. Maruyama, M. Krause, and J. Karn. 1989. Sequence analysis of the complete *Caenorhabditis elegans* myosin heavy chain gene family. *J. Mol. Biol.* **205**: 603–613.
- Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang, A.E. Kilburn, W.-H. Lee, and S.J. Elledge. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* **7**: 555–569.
- Farrar, M.A., J. Alberola-Ila, and R.M. Perlmutter. 1996. Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. *Nature* **383**: 178–181.
- Fields, S. and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature* **340**: 245–246.
- Finney, M. and G. Ruvkun. 1990. The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**: 895–905.
- Fire, A., S.W. Harrison, and D. Dixon. 1990. A modular set of *lacZ* fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**: 189–198.
- Fire, A., D. Albertson, S.W. Harrison, and D.G. Moerman. 1991. Production of antisense RNA leads to effective and specific inhibition of gene expression in *C. elegans* muscle. *Development* **113**: 503–514.
- Goshima, Y., F. Nakamura, P. Strittmatter, and S.M. Strittmatter. 1995. Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* **376**: 509–514.
- Guan, K. and J.E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**: 262–267.
- Guo, S. and K.J. Kemphues. 1996. A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**: 455–458.
- Hedgecock, E.M., J.G. Culotti, J.N. Thomson, and L.A. Perkins. 1985. Axonal guidance mutants of *Caenorhabditis elegans* identified by filling sensory neurons with fluorescein dyes. *Dev. Biol.* **111**: 158–170.
- Hedgecock, E.M., J.G. Culotti, D.H. Hall, and B.D. Stern. 1987. Genetics of cell and axon migrations in *Caenorhabditis elegans*. *Development* **100**: 365–382.
- Hedgecock, E.M., J.G. Culotti, and D.H. Hall. 1990. The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **2**: 61–85.
- Hill, K.K., V. Bedian, J.-L. Juang, and F.M. Hoffmann. 1995. Genetic interactions between the *Drosophila* Abelson (Abl) tyrosine kinase and failed axon connections (fax), a novel protein in axon bundles. *Genetics* **141**: 595–606.
- Huang, L.S., P. Tzou, and P.W. Sternberg. 1994. The *lin-15* locus encodes two negative regulators of *Caenorhabditis elegans* vulval development. *Mol. Biol. Cell* **5**: 395–412.
- Huang, X.-Y. and D. Hirsh. 1989. A second trans-spliced RNA leader sequence in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci.* **86**: 8640–8644.
- Ishii, N., W.G. Wadsworth, B.D. Stern, J.G. Culotti, and E.M. Hedgecock. 1992. UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. *Neuron* **9**: 873–881.
- Keino-Masu, K., M. Masu, L. Hinck, E.D. Leonardo, S.S.-Y. Chan, J.G. Culotti, and M. Tessier-Lavigne. 1996. Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* **87**: 175–185.
- Kim, Y.-T. and C.-F. Wu. 1987. Reversible blockage of neurite development and growth cone formation in neuronal cultures of a temperature-sensitive mutant of *Drosophila*. *J. Neurosci.* **7**: 3245–3255.
- Koenig, J.H., K. Saito, and K. Ikeda. 1983. Reversible control of synaptic transmission in a single gene mutant of *Drosophila melanogaster*. *J. Cell Biol.* **96**: 1517–1522.
- Kosaka, T. and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster*, *shibire^{ts1}*. *J. Cell Biol.* **97**: 499–507.

- Kramer, J.M., R.P. French, E. Park, and J.J. Johnson. 1990. The *Caenorhabditis elegans* *rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. *Mol. Cell. Biol.* **10**: 2081–2089.
- Leung-Hagesteijn, C., A.M. Spence, B.D. Stern, Y. Zhou, M.-W. Su, E.M. Hedgecock, and J.G. Culotti. 1992. UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domain, guides cell and pioneer axon migrations in *C. elegans*. *Cell* **71**: 289–299.
- Li, W., R.K. Herman, and J.E. Shaw. 1992. Analysis of the *Caenorhabditis elegans* axonal guidance and outgrowth gene *unc-33*. *Genetics* **132**: 675–689.
- Link, C.D., M.A. Silverman, M. Breen, K.E. Watt, and S.A. Dames. 1992. Characterization of *Caenorhabditis elegans* lectin-binding mutants. *Genetics* **131**: 867–881.
- Luo, Z., G. Tzivion, P.J. Belshaw, D. Vavvas, M. Marshall, and J. Avruch. 1996. Oligomerization activates c-RAF-1 through a Ras-dependent mechanism. *Nature* **383**: 181–185.
- Manser, E., T. Leung, H. Salihuddin, Z. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**: 40–46.
- McIntire, S.L., G. Garriga, J. White, D. Jacobson, and H.R. Horvitz. 1992. Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* **8**: 307–322.
- Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**: 3959–3970.
- Ogura, K., C. Wicky, L. Magnenat, H. Tobler, I. Mori, F. Müller, and Y. Ohshima. 1994. *Caenorhabditis elegans* *UNC-51* gene required for axonal elongation encodes a novel serine/threonine kinase. *Genes & Dev.* **8**: 2389–2400.
- Otsuka, A.J., R. Franco, B. Yang, K.H. Shim, L.Z. Tang, Y.Y. Zhang, P. Boontrakulpoontawee, A. Jeyaparakash, E. Hedgecock, and V.I. Wheaton. 1995. An ankyrin-related gene (*unc-44*) is necessary for proper axonal guidance in *Caenorhabditis elegans*. *J. Cell. Biol.* **129**: 1081–1092.
- Pierceall, W.E., M.A. Reale, A.F. Candia, C.V. Wright, K.R. Cho, and E.R. Fearon. 1994. Expression of a homologue of the deleted in colorectal cancer (DCC) gene in the nervous system of developing *Xenopus* embryos. *Dev. Biol.* **166**: 654–665.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Serafini, T., T.E. Kennedy, M.J. Galko, C. Mirzayan, T.M. Jessell, and M. Tessier-Lavigne. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78**: 409–424.
- Shibuya, H., K. Yamaguchi, K. Shirakabe, A. Tonegawa, Y. Gotoh, N. Ueno, K. Irie, E. Nishida, and K. Matsumoto. 1996. TAB1: An activator of the TAK1 MAPKKK in TGF- β signal transduction. *Science* **272**: 1179–1182.
- Siddiqui, S.S. 1990. Mutations affecting axonal growth and guidance of motor neurons and mechanosensory neurons in the nematode *Caenorhabditis elegans*. *Neurosci. Res. Suppl.* **13**: S171–S190.
- Simske, J.S., S.M. Kaech, S.A. Harp, and S.K. Kim. 1996. LET-23 receptor localization by the cell junction protein LIN-7 during *C. elegans* vulval induction. *Cell* **85**: 195–204.
- Starich, T.A., R.Y.N. Lee, C. Panzarella, L. Avery, and J.E. Shaw. 1996. *eat-5* and *unc-7* represent a multigene family in *Caenorhabditis elegans* involved in cell–cell coupling. *J. Cell. Biol.* **134**: 537–548.
- Wadsworth, W.G., H. Bhatt, and E.M. Hedgecock. 1996. Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in *C. elegans*. *Neuron* **16**: 35–46.
- Wood, W.B. 1988. *The nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Yasukawa, T., C. Kanei-Ishii, T. Maekawa, J. Fujimoto, T. Yamamoto, and S. Ishii. 1995. Increase of solubility of foreign proteins in *Escherichia coli* by co-expression of the bacterial thioredoxin. *J. Biol. Chem.* **270**: 25328–25331.

The UNC-14 protein required for axonal elongation and guidance in *Caenorhabditis elegans* interacts with the serine/threonine kinase UNC-51.

K Ogura, M Shirakawa, T M Barnes, et al.

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