The autophagy-related kinase UNC-51 and its binding partner UNC-14 regulate the subcellular localization of the Netrin receptor UNC-5 in Caenorhabditis elegans

Ken-ichi Ogura1,4,* and Yoshio Goshima1,2

UNC-51 and UNC-14 are required for the axon guidance of many neurons in Caenorhabditis elegans. UNC-51 is a serine/threonine kinase homologous to yeast Atg1, which is required for autophagy. The binding partner of UNC-51, UNC-14, contains a RUN domain that is predicted to play an important role in multiple Ras-like GTPase signaling pathways. How these molecules function in axon guidance is largely unknown. Here we observed that, in unc-51 and unc-14 mutants, UNC-5, the receptor for axon-guidance protein Netrin/UNC-6, abnormally localized in neuronal cell bodies. By contrast, the localization of many other proteins required for axon guidance was undisturbed. Moreover, UNC-5 localization was normal in animals with mutations in the genes for axon guidance proteins, several motor proteins, vesicle components and autophagy-related proteins. We also found that unc-5 and unc-6 interacted genetically with unc-51 and unc-14 to affect axon guidance, and that UNC-5 co-localized with UNC-51 and UNC-14 in neurons. These results suggest that UNC-51 and UNC-14 regulate the subcellular localization of the Netrin receptor UNC-5, and that UNC-5 uses a unique mechanism for its localization; the functionality of UNC-5 is probably regulated by this localization.

KEY WORDS: Caenorhabditis elegans, Axon guidance, Autophagy, Netrin, Kinase

INTRODUCTION

Newborn neurons extend axons to precise targets (other neurons or muscle), and form synapses on them. Growing axons receive guidance information at the growth cones, a specialized structure at the growing axonal tip of axons (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001; Dickson, 2002). Many of the molecules responsible for axon pathfinding during development have been identified – however, little is known about how these molecules are regulated to generate appropriate connections.

The axon guidance molecule Netrin was originally identified in Caenorhabditis elegans as UNC-6 (Ishii et al., 1992). Some axons are attracted to Netrin, but others are repelled by it (Hedgecock et al., 1990; McIntire et al., 1992; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). In C. elegans, Netrin/UNC-6 is secreted from ventral cells (Wadsworth et al., 1996), and it is thought to form a ventral-to-dorsal concentration gradient (Wadsworth, 2002). Two C. elegans Netrin receptors, UNC-5 and UNC-40 (mammalian DCC homolog), are known (Chan et al., 1996; Leung-Hagesteijn et al., 1992). Both belong to the immunoglobulin superfamily, have a single transmembrane domain and are expressed in neurons that respond to Netrin (Chan et al., 1996; Su et al., 2000). Both UNC-5 and UNC-40 are required for dorsally extending axons to be repulsed by ventral Netrin (Hedgecock et al., 1990; McIntire et al., 1992). However, the ventrally extending axons are attracted to ventral Netrin, and require only UNC-40 for this response. In mammals, Netrin binds the Ig domain of UNC-5, and the fibronectin type III domain of UNC-40/DCC (Kruger et al., 2004).

In the unc-51 and unc-14 C. elegans mutants, there are many neurons with guidance defects (Hedgecock et al., 1985; McIntire et al., 1992). In addition, abnormal membrane structures (e.g. abnormally large varicosities or cisternae-like structures) have been observed in their axons. UNC-51 is a serine/threonine kinase homologous to yeast Atg1, which is required for autophagy, a form of catabolic vesicle trafficking (Ogura et al., 1994; Matsuura et al., 1997; Straub et al., 1997). UNC-14 is a novel protein that contains a RUN domain (Ogura et al., 1997). While the function of the RUN domain in UNC-14 is not known, RUN domains are predicted to play important roles in the Rap and Raf family GTPase signaling pathways that affect vesicle trafficking (Callebaut et al., 2001). UNC-51 and UNC-14 are expressed in many neurons, and UNC-51 directly binds UNC-14; however, their molecular functions on axon guidance are largely unknown.

Here, we report that UNC-51 and UNC-14 regulate the subcellular localization of the Netrin receptor UNC-5. We propose that UNC-5 uses a unique localization mechanism, which probably regulates its function.

MATERIALS AND METHODS

Worms

Bristol strain N2 was used as the standard wild-type strain. The worms were handled as described by Brenner (Brenner, 1974). The mutants used in this paper are shown below:

| LG I: unc-14(e57), unc-73(e936), unc-40(n324), unc-11(e47), htk2(qs48) and unc-101(m1); |
| LG II: unc-126(e919), ref-3(pk1426) and js676(unc-25p::gfp); |
| LG III: at2(qs48); |
| LG IV: unc-5(e53), unc-44(e362), unc-33(mn407), jds111(unc-25p::smub-1::gfp) and nux24(gbr-1::gfp); |
| LG V: unc-51(e569), unc-51(k58::Tc1) and unc-51(k59); |
| LG X: lin-15(n765ts), unc-6(ju152), unc-6(ev400), kypsy156(str-1p::odr-10::gfp), nus9(unc-5::gfp) and osli12(unc-47p::gfp). |

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Molecular analysis
The unc-25 promoter region (Jin et al., 1999) was amplified by PCR from wild-type genomic DNA using KOD-Plus (TOYOBO). The unc-25 promoter region was cloned into the GFP expression vector pPD95.77. The resultant plasmid p77-u25KP was used to express several GFP fusion proteins in DD/VD neurons. We also used monomeric red fluorescent protein (mRFP) for double labeling (Campbell et al., 2002). To construct mRFP fusion proteins, the GFP-coding region of pPD95.77 was swapped with the mRFP ORF. The unc-25 promoter region was cloned into the resulting plasmid. This plasmid (pu25-mR1) was used to express several mRFP fusion proteins. Full-length ORFs were amplified by PCR from EST clones or RT-PCR products. The EST clones used for the analysis were yk668c8 (unc-51), yk459e7 (unc-14), yk678e6 (unc-115), yk484d2 (unc-40), yk344c5 (mig-2), yk643f1 (ced-10), yk622a3 (rab-5), yk648d7 (rab-7), yk66d6 (rab-11) and yk281f2 (lgg-1). The EST clones were kindly provided by Y. Kohara.

Transformation of C. elegans
Transformation was performed as described by Mello et al. (Mello et al., 1991). MT8189 [lin15(n765ts)] X was used as the transformation strain, and pJM23 was used as the transformation marker. For the expression analysis, each sample of 10–50 ng/μl DNA and pJM23 was injected into the adult gonad (final concentration of DNA=100 ng/μl).

RNA-mediated interference
EST clones yk668c8 (unc-51/ATG1), yk656b6 (bec-1=ATG6), yk281g8 (M7.5=ATG7), yk281f2 (lgg-1=ATG8) and yk515b7 (F41E6.13=ATG18) were used as templates for double-stranded RNA synthesis. Although RNAi is not generally effective in C. elegans neural cells, rrf-3 mutants are hypersensitive to RNAi (Sijen et al., 2001), and RNAi can be used in these mutants very effectively to silence at least axon guidance genes (e.g. unc-51, unc-14, unc-33, max-1 and unc-73, and data not shown). We used rrf-3(pk1426) mutants for our all RNAi analysis.

For the injection method, using the PCR-amplified insert, both RNA strands were simultaneously synthesized with T3 and T7 RNA polymerase (Promega), and the RNA mixture was heat-denatured and annealed to form double-stranded RNA. Microinjection of the RNA was performed as described (Mello et al., 1991; Fire et al., 1998) at a concentration of 3–4 mg/ml in TE. For the feeding method (Kamath et al., 2000), PCR-amplified inserts were cloned into pPD129.36 at the I site. HT115(DE3) was used for the host strain.

Visualization of DD/VD axons
DD/VD neurons were visualized by using otxIs12 (=unc-47p::gfp) (McIntire et al., 1997) or jds76 (=unc-25p::gfp) (Huang et al., 2002). To analyze the axon guidance defects in mutants, we counted the number of DD/VD axons that could reach the dorsal nerve cord in L4 larvae or young adults. At least 20 worms were examined and averaged for each strain.

Imaging analysis
An LSM510 confocal microscope (Zeiss) was used to collect the images.

RESULTS
The Netrin receptor UNC-5 accumulates in neuronal cell bodies in unc-51 and unc-14 mutants
The DD/VD neurons of C. elegans are GABAergic motoneurons (Fig. 1A,B) (McIntire et al., 1992), and their cell bodies are located ventrally (White et al., 1986). During axon growth, they first extend their axons anteriorly, but, after some extension, their axons branch into a short anterior branch and a dorsally extending branch. When the dorsally extending branch reaches the dorsal nerve cord, they branch again, with a short branch extending anteriorly and a longer one extending posteriorly. Many genes required for DD/VD axon guidance have been identified (McIntire et al., 1992; Forrester and Garriga, 1997; Huang et al., 2002; Wu et al., 2002; Lundquist et al., 2001).

We speculated that UNC-5 and UNC-14 might regulate the localization of molecules required for DD/VD axon guidance, probably by vesicle trafficking, for the following reasons: (1) mutants of unc-51 and unc-14 have defects in DD/VD axon guidance (McIntire et al., 1992) (Fig. 1C,D); (2) Atg1, a yeast homolog of UNC-51, is required for autophagy (Matsuura et al., 1997; Straub et al., 1997); (3) UNC-14 has a RUN domain, which is predicted to play important roles in the Rap and Rab family GTPase signaling pathways for vesicle trafficking (Callebaut et al., 2001); (4) unc-51 and unc-14 are expressed in DD/VD neurons (Ogura et al., 1997).

We first investigated the localization of the Netrin receptor UNC-5 in DD/VD neurons, because their mutant phenotypes are similar to those in unc-51 and unc-14 (McIntire et al., 1992) (Fig. 1F), and UNC-5 is expressed in the DD/VD neurons (Su et al., 2000). In wild-type animals, functional UNC-5::GFP, which can rescue the guidance defects of unc-5, is found associated with small vesicles that are uniformly distributed in axons and cell bodies (Killeen et al., 1999) (Fig. 2B). In unc-51, UNC-5::GFP accumulated in the neuronal cell bodies (Fig. 2B, Fig. 3B), but small amounts of UNC-5::GFP were found in the axons (Fig. 2B). We examined UNC-
5::GFP in unc-14 mutants, and observed the similar abnormal accumulation in neuronal cell bodies (Fig. 2D, Fig. 3C). The accumulation of UNC-5::GFP was never observed in wild type but commonly observed in the unc-51 and unc-14 mutants.

We quantified the GFP fluorescence in the cell bodies and axons (Fig. 2F,G). In unc-51(e369) mutants, large amounts of UNC-5::GFP accumulated in the cell bodies compared with those of wild type (Fig. 2F), but small amounts of UNC-5::GFP were found in the axons (Fig. 2G). In unc-14(e57) mutants, the fluorescence quantity in the cell bodies and axons was not statistically different from that of wild type (Fig. 2F,G), suggesting that the amount of UNC-5::GFP was not affected. In unc-14(e57) mutants, the UNC-5::GFP vesicles appeared to abnormally accumulate at a certain region in the cell bodies (Fig. 3C).

In the unc-51 and unc-14 mutants, cell-body accumulation UNC-5::GFP was observed in many neurons that normally express UNC-5 throughout development and adulthood (data not shown). Interestingly, the distribution of UNC-5::GFP seemed normal in non-neural distal tip cells (DTCs) (Fig. 4A-C) and excretory cells (Fig. 4D-F) in these mutants, suggesting that UNC-51 and UNC-14 regulate the subcellular localization of UNC-5 only in neurons.

**UNC-51 and UNC-14 do not generally regulate the localization of proteins in neurons**

We next examined the localization of another Netrin receptor, UNC-40, in unc-51 and unc-14 mutants. UNC-40 is also required for the axon guidance of the DD/VD neurons (Fig. 4G). In wild-type animals, functional UNC-40::GFP is localized to the cell surface of DD/VD neurons (Chan et al., 1996) (Fig. 4H); this was a different pattern to that of UNC-5::GFP (small vesicles, Fig. 2A). The UNC-40::GFP localization in unc-51 and unc-14 mutants appeared to be normal (Fig. 4I,J).

There are many genes required for DD/VD axon guidance. To analyze whether or not UNC-51 regulates the localization of proteins in DD/VD neurons generally, we examined the localization of UNC-33 (CRMP) (Li et al., 1992), UNC-76 (FEZ) (Bloom and Horvitz, 1997), UNC-115 (ablLIM) (Lundquist et al., 1998), UNC-73 (Trio) (Steven et al., 1998), CED-10 (Rac) (Reddien and Horvitz, 2000) and MIG-2 (Rac) (Zipkin et al., 1999), which are required for DD/VD axon guidance. We also examined the localization of VAB-8 (Wolf et al., 1998), which is required for posterior axon outgrowth. We found their localization were normal in the unc-51 and unc-14 mutants (Table 1).

We next examined the localization of ODR-10 (an odorant receptor) (Sengupta et al., 1996) in the AWB neurons, and GLR-1 (AMPA-class glutamate receptor) (Maricq et al., 1995; Hart et al., 1995) in several interneurons of the head (Table 1). Their localizations were also normal in the unc-51 and unc-14 mutants. We also examined the localization of RAB-11 (recycling endosome marker) (Grant and Hirsh, 1999) and LGG-1 (autophagosome marker) (Melendez et al., 2003), in DD/VD neurons. Their localization was not affected in the unc-51 and unc-14 mutants. As abnormally large synapses are observed in unc-51 and unc-14 mutants (McIntire et al., 1992; Crump et al., 2001), we examined the SNB-1::GFP localization in these mutants. We found abnormal accumulation of SNB-1::GFP in the DD/VD cell bodies of unc-51 and unc-14 mutants (Table 1).

These results suggest that UNC-51 and UNC-14 regulate the localization of UNC-5 and SNB-1, at least in regard to its accumulation in the cell body, but do not appear to regulate the localization of proteins generally in neurons.

**Localization of UNC-5::GFP is normal in other guidance mutants, autophagy-related mutants, kinesin mutants and clathrin adaptor mutants**

To distinguish the UNC-5 accumulation in unc-51 and unc-14 mutants from secondary defects caused by the abnormal path-finding of the DD/VD neurons, we examined the localization of UNC-5::GFP in other mutants with guidance defects in the DD/VD neurons, including unc-73, unc-44, unc-33, unc-40, unc-6 and maxi-1 (Table 2) and found that the UNC-5::GFP localization was normal.
These results suggest that the accumulation of UNC-5::GFP in neuronal cell bodies was not a secondary effect of improper axon guidance.

Because UNC-51 is a homolog of Atg1, which is required for yeast autophagy, we investigated whether there was a connection between the accumulation phenotype and autophagy. To do this, we examined the effect of autophagy-related genes on the localization of UNC-5::GFP. We tested unc-5(e369), M7.5/ATG7, lgg-1/ATG8 and F41E6.13/ATG18, which are required (as is unc-51/ATG1) for autophagy in C. elegans (Melendez et al., 2003). Interestingly, UNC-5::GFP localization was normal in these RNAi worms (Table 2). In addition, we did not find any obvious co-localization of UNC-5::GFP and the autophagosome marker GFP::LGG-1 (data not shown). We concluded that conventional autophagy was not involved in the accumulation of UNC-5.

In yeast, Atg1 is required for both cytoplasm-to-vacuole targeting (Cvt) and autophagy. The Atg1 kinase activity is required only for Cvt but not for autophagy (Abeliovich et al., 2003). As unc-51(e369) has a mutation at the C-terminal region (probable regulatory region), the kinase domain is intact. One possibility is that, in unc-51(e369) mutants, the kinase activity of UNC-51 may be unregulated by the mutation at the probable regulatory region, resulting in abnormal Cvt. If this possibility is correct, unc-51(RNAi) should show different phenotypes to those of unc-51(e369). In addition, RNAi to unc-51(e369) mutants should rescue the phenotypes. We found that unc-51(RNAi) showed an unc-51-like phenotype with respect to UNC-5 vesicles (data not shown). We also found that unc-51(RNAi) in the unc-51(e369) mutant made the guidance defects more severe: the number of axons reaching the nerve cord in unc-51(369); control (RNAi) hermaphrodites was 5.9, whereas in unc-51(e369); unc-51(RNAi) hermaphrodites it was 3.8 (P<0.01, Bonferroni correction). These analyses indicate that the phenotypes observed in unc-51(e369) do not result from unregulated UNC-51 (abnormal Cvt).

We next examined the localization of UNC-5::GFP in mutants of motor proteins or vesicle components, including kinesin mutants (unc-104 and unc-116) (Otsuka et al., 1991; Patel et al., 1993) and clathrin adapter mutants (unc-101 and unc-11) (Lee et al., 1994; Nonet et al., 1999). UNC-5::GFP localization was also normal in these mutants (Table 2), suggesting that these proteins are not required for its localization.

Genetic interaction among unc-5, unc-6, unc-51 and unc-14

To elucidate the mechanism of accumulation of UNC-5 in the DD/VD neural cell bodies in unc-51 and unc-14 mutants, we looked for genetic interactions among unc-5, unc-6, unc-51 and unc-14 that would affect DD/VD axon guidance. First, we examined genetic interactions between unc-5 and unc-51, and between unc-6 and unc-51. unc-5(e53) is a null allele in which 100% of the axons are prevented from reaching the dorsal nerve cord (Fig. 1F, Fig. 5A). As e53 is a recessive mutant, e53/+ heterozygotes are identical to wild-type animals (Fig. 5A, e53/+). Using the unc-5(e53) heterozygotes, we found that the low dose of unc-5 strongly enhanced the defects of both unc-51 and unc-14 (Fig. 5A). These results indicate that unc-5 interacts genetically with unc-51 and unc-14 and are consistent with our hypothesis that UNC-51 and UNC-14 regulate the localization of UNC-5. Overexpression of unc-5 in unc-51 or unc-14 mutants had no effect on the guidance defects (data not shown).

We next examined the effect of genetic interactions between Netrin/unc-6 and unc-51, and between Netrin/unc-6 and unc-14 on DD/VD axon guidance. unc-6(ev400) is a null allele in which 100% of the axons are prevented from reaching the dorsal nerve cord (Fig. 1E; Fig. 5B). unc-6(ju152) is a very weak allele, and only 15.0% of the axons were blocked (Fig. 5B, wild type: 5.6%). We found that unc-6(ju152) strongly enhanced the defects of both unc-51 and unc-14 (Fig. 5B), suggesting that unc-6 interacts genetically with unc-51 and unc-14.

We examined the effect of genetic interactions between another Netrin receptor/unc-40 and unc-51 on DD/VD axon guidance. unc-40(n324) is a null allele in which 80% of the axons are prevented from reaching the dorsal nerve cord (Fig. 4G, Fig. 5C). As n324 is a recessive mutant, n324/+ heterozygotes are identical...
localization of UNC-40 was normal in these mutants. We tried identifying the localization signal that targeted UNC-5 to small vesicles.

First, we found that neither the extracellular nor cytoplasmic domain was essential for the localization to small vesicles, as deletion of these domains did not affect it (Fig. 6B,J,C,L). In order to avoid the effect of endogenous UNC-5 in our experiments, we also examined the deleted UNC-5 localization in unc-51(e53) null mutants. The results were identical to those in a wild-type background (data not shown), suggesting that these deleted UNC-5 proteins were capable of localizing at small vesicles by themselves.

We next examined the importance of the transmembrane domain of UNC-5, and found that it was essential for the localization, as the deletion of the transmembrane domain completely abolished the localization of UNC-5 to small vesicles (Fig. 6D,N,E,O). We examined the localization of the transmembrane domain alone, and found that it did not localize to small vesicles at all (Fig. 6F,P). These results suggest that the targeting of UNC-5 to small vesicles is regulated redundantly by the extracellular and cytoplasmic domains.

As UNC-5 does not have any obvious N-terminal hydrophobic signal sequence for insertion into the membrane, the lack of the signal sequence could result in its unique localization to small vesicles. We tried adding the signal sequence from UNC-40 to UNC-5 proteins were capable of localizing at small vesicles by themselves.

We next examined the effect of genetic interactions between unc-51 and unc-14 on DD/VD axon guidance. unc-51(e369) is a severe allele and unc-14(e57) is a null allele. We found that the phenotypes of the double mutant unc-51(e369); unc-14(e57) were identical to those of unc-51(e369) (Fig. 5A), indicating that unc-51 and unc-14 function in the same genetic pathway. This result is consistent with our previous work showing that UNC-51 directly binds UNC-14. UNC-51 and UNC-14 probably cooperate to regulate the localization of UNC-5 in DD/VD neurons.

The combination of the transmembrane and cytoplasmic domains are necessary and sufficient for the regulation by UNC-51

We thought that the association of UNC-5 with small vesicles might be related to its abnormal cell-body accumulation in unc-51 and unc-14 mutants, given that the cell-surface localization of UNC-40 was normal in these mutants. To test this idea, we performed these analyses using GFP-fusion proteins. In these assays, we examined whether or not the proteins accumulated in the cell bodies.

Table 1. Protein accumulation at cell bodies in unc-51 and unc-14 mutants

<table>
<thead>
<tr>
<th>Proteins</th>
<th>unc-51(e369)</th>
<th>unc-14(e57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNC-5 (Netrin receptor)</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>UNC-40 (Netrin receptor)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UNC-44 (ankyrin)*</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>UNC-33 (CRMP)†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UNC-115 (ablLIM)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UNC-76 (FEZ1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CED-10 (Rac)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MIG-2 (Rac)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UNC-73 (TriO/GEF)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VAB-8 (kinasin)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LGG-1 (Atg8/LC3)</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>RAB-11 (RAB 11)</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>UNC-14 (RUN domain)</td>
<td>Weak‡</td>
<td>ND</td>
</tr>
<tr>
<td>UNC-51 (S/T kinase)</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>IDA-1 (ICAS12)²</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SNB-1 (synaptobrevin)³</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ODR-10 (odorant receptor)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GLR-1 (AMPA receptor)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

In DTC or excretory cells

| UNC-5 (Netrin receptor) | –             | –           |

We performed these analyses using GFP-fusion proteins. In these assays, we examined whether or not the proteins accumulated in the cell bodies.

Table 2. Accumulation of UNC-5::GFP in DD/VD cell bodies

<table>
<thead>
<tr>
<th>Mutants</th>
<th>UNC-5::GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>unc-51(e369)</td>
<td>++</td>
</tr>
<tr>
<td>unc-51(kls38::Tc1)</td>
<td>+</td>
</tr>
<tr>
<td>unc-14(e57)</td>
<td>+</td>
</tr>
<tr>
<td>unc-6(e400)</td>
<td>–</td>
</tr>
<tr>
<td>unc-40(n324)</td>
<td>–</td>
</tr>
<tr>
<td>unc-73(e936)</td>
<td>–</td>
</tr>
<tr>
<td>unc-44(e362)</td>
<td>–</td>
</tr>
<tr>
<td>unc-33(mn407)</td>
<td>–</td>
</tr>
<tr>
<td>max-1(RNAi)*</td>
<td>–</td>
</tr>
<tr>
<td>unc-104(e1265)</td>
<td>–</td>
</tr>
<tr>
<td>unc-116(e2310)</td>
<td>–</td>
</tr>
<tr>
<td>unc-11(e47)</td>
<td>–</td>
</tr>
<tr>
<td>unc-101(m1)</td>
<td>–</td>
</tr>
<tr>
<td>bs-1(1ATG6(RNAi)*</td>
<td>–</td>
</tr>
<tr>
<td>M7.5(ATG7(RNAi)*</td>
<td>–</td>
</tr>
<tr>
<td>lgg-1(1ATG8(RNAi)*</td>
<td>–</td>
</tr>
<tr>
<td>F41E6.13(AT1G18(RNAi)*</td>
<td>–</td>
</tr>
</tbody>
</table>

*RNAi analysis was performed using nfi-3(pk1426) worms, which are hypersensitive to RNAi.

We finally examined the effect of genetic interactions between unc-51 and unc-14 mutants. We think that UNC-51 and UNC-14 regulate the localization of the signal sequence that targeted UNC-5 to small vesicles.

We performed these analyses using GFP-fusion proteins. In these assays, we examined whether or not the proteins accumulated in the cell bodies.

As UNC-5 does not have any obvious N-terminal hydrophobic signal sequence for insertion into the membrane, the lack of the signal sequence could result in its unique localization to small vesicles. We tried adding the signal sequence from UNC-40 to UNC-5.
UNC-5 and UNC-51 or UNC-5 and UNC-14 are partly co-localized in DD/VD neurons

We next examined the co-localization of UNC-5, UNC-51 and UNC-14 in DD/VD neurons. UNC-51::GFP and UNC-14::GFP often appeared as puncta in the axons or cell bodies (Fig. 7A,D). We found that UNC-51::GFP and UNC-5:mRFP or UNC-14::GFP and UNC-5::mRFP were partly co-localized in DD/VD neurons (Fig. 7C,F, arrowheads).

To discover if there was any physical interaction between UNC-5 and UNC-51, or between UNC-5 and UNC-14, we tried using the yeast two-hybrid assay. However, we did not find evidence of any physical interaction between the UNC-5 cytoplasmic region and UNC-51, UNC-14. These results suggest that the regulation of UNC-5 localization by UNC-51 and UNC-14 may be indirect or involve very weak or transient physical interactions (for example phosphorylation).

DISCUSSION

In this paper, we have shown that: (1) in unc-51 and unc-14 mutants, the Netrin receptor UNC-5 localized abnormally in the cell bodies of DD/VD neurons; (2) many proteins required for axon guidance had normal expression patterns of UNC-5; (3) UNC-5 localization was normal in many mutants, including those of guidance proteins, several motor proteins, vesicle components and autophagy-related proteins; (4) low-dose unc-5 strongly enhanced the defects of unc-51 and unc-14; (5) a very weak mutation of unc-6/Netrin strongly enhanced the defects of unc-51 and unc-14; (6) low-dose unc-40 did not enhance the defects of unc-51; (7) the unc-14 mutation did not enhance the defects of unc-51; (8) both of the transmembrane and cytoplasmic regions of UNC-5 were required for the accumulation in unc-51(e369) mutants; (9) UNC-5, UNC-51 and UNC-14 partly co-localized in the DD/VD neurons.

UNC-51 and UNC-14 regulate the proper localization of UNC-5 in neurons by an unknown mechanism

We have shown that, in unc-51 and unc-14 mutants, the Netrin receptor UNC-5 accumulated in the cell bodies of DD/VD neurons. Cell-body accumulations of UNC-5::GFP were also observed in many neurons that express UNC-5 at all stages of development and adulthood (data not shown). This UNC-5::GFP accumulation did not result as a secondary effect of the misrouted axons, as other guidance mutants (unc-73, unc-44, unc-33, etc.) have normal localization of UNC-5::GFP in DD/VD neurons. A simple explanation is that UNC-51 and UNC-14 regulate the subcellular localization of UNC-5. We have also shown that the localization of other GFP fusion proteins (UNC-76, UNC-44, UNC-33, etc.) was normal in unc-51 and unc-14 mutants, except for SNB-1, suggesting that UNC-51 and UNC-14 regulate the localization of UNC-5 and SNB-1.
In unc-51 and unc-14 mutants, we did not find an abnormal accumulation of UNC-5::GFP in non-neural cells (DTCs or excretory cells), suggesting that UNC-51 and UNC-14 regulate the localization of UNC-5 only in neurons. These results are consistent with the fact that guidance defects are not observed in non-neuronal cells (DTCs or excretory cells) in unc-51 or unc-14 mutants.

We found that UNC-5::GFP localization was also normal in worms that received injections of the RNAi for autophagy-related genes (bec-1/ATG6, M7.5/ATG7, lgg-1/ATG8 and F41E6.13/ATG18), kinesin mutants (unc-104 and unc-116) and clathrin adaptor mutants (unc-101 and unc-11). We think the proper localization of UNC-5::GFP is mediated by an unknown mechanism through UNC-51 and UNC-14 (see below).

UNC-51 and UNC-14 cooperate to regulate the localization of UNC-5

We have shown that unc-5 and unc-6 interacted genetically with unc-51 and unc-14 to affect DD/VD axon guidance, as low-dose unc-5 or a very weak allele of unc-6 strongly enhanced the defects of unc-51 and unc-14. In addition, UNC-5, UNC-51 and UNC-14 partly co-localized in the DD/VD neurons. These are consistent with our hypothesis that UNC-51 and UNC-14 regulate the subcellular localization of UNC-5. From these findings, we concluded that the unc-51 and unc-14 defects in the dorsal axon guidance of the DD/VD neurons is explained at least in part by the abnormal localization of UNC-5. That is, the abnormal localization of UNC-5 in the DD/VD neurons in unc-51 and unc-14 mutants probably leads to a paucity of UNC-5 at the cell membrane of growth cones, so that they respond weakly if at all to Netrin/UNC-6, resulting in abnormal axon guidance.

We have also shown that the phenotypes of unc-51(e369); unc-14(e57) double mutants were identical to those of unc-51(e369), indicating that unc-51 and unc-14 function in the same genetic pathway. This is consistent with our previous data that UNC-51 can bind UNC-14 directly. We think that UNC-51 and UNC-14 cooperate to regulate the localization of UNC-5.

The transmembrane and cytoplasmic regions are important for regulation by UNC-51

We have shown that none of the major protein domains of UNC-5, the extracellular, transmembrane or cytoplasmic domains, was sufficient to target UNC-5 to small vesicles. The
combination of the extracellular domain and the transmembrane domain, or of the transmembrane domain and the cytoplasmic domain, was sufficient for this targeting. From these results, we conclude that the localization of UNC-5 to small vesicles is probably regulated redundantly by the extracellular and cytoplasmic domains.

We have shown that the combination of the transmembrane domain and the cytoplasmic domain of UNC-5 was necessary and sufficient for the accumulation in unc-51(e369) mutants. These results suggest that these domains probably include a regulatory region controlled by UNC-51 (and UNC-14) for the UNC-5 localization. As UNC-51 and UNC-14 are cytoplasmic proteins, we predicted that they might physically interact with the cytoplasmic region of UNC-5. However, we did not detect a physical interaction between the cytoplasmic region of UNC-5 and UNC-51 or UNC-14 in our two-hybrid analysis (data not shown). We think that the regulation of UNC-5 localization by UNC-51 and UNC-14 is probably indirect or through a very weak or transient physical interaction (for example, phosphorylation). We cannot exclude a possibility that the transmembrane domain of UNC-5 may be required for their physical interaction.

**Possible function of UNC-51 and UNC-14 in UNC-5 localization**

Our working model of the localization of the Netrin receptor UNC-5 is shown in Fig. 8A. In our model, UNC-5 is associated with a small vesicle that is transported to the axon by an unknown mechanism. UNC-51 and UNC-14 cooperate to regulate the formation, selection or transport of the vesicle.

For the reasons given below, we think that UNC-51 and UNC-14 may cooperate with unknown motor proteins to regulate the transport of UNC-5. (1) Synaptic vesicle components are normally localized in the kinesin mutants unc-104 and unc-116 (Byrd et al., 2001; Sakamoto et al., 2005). UNC-14 cooperates with kinesin UNC-116 for synaptic vesicle transport. (2) UNC-51 can bind VAB-8, a kinesin-like protein, and phosphorylate VAB-8 in vitro (Wolf et al., 1998; Lai and Garriga, 2004). Overexpression of vab-8 partially suppresses the guidance defects of unc-51 mutants seen in the posterior outgrowth of CAN. Although VAB-8 probably cooperates with UNC-51 for the posterior outgrowth of CAN, we think that VAB-8 is unlikely to regulate UNC-5 localization, because the dorsal axon guidance that requires UNC-5 is normal in vab-8 mutants (Wightmann et al., 1996). There are 21 kinesins, six dyneins and 17 myosins in the C. elegans genome (The C. elegans Sequencing Consortium, 1998). An unknown motor protein may function with UNC-51 and UNC-14 to regulate UNC-5 localization.

In mammals, UNC51.1, a mouse homolog of UNC-51, binds SynGAP and Syntenin, and their complex regulates Rab5-mediated endocytosis in the formation of parallel fibers in cerebellar granule neurons (Tomoda et al., 1999; Tomoda et al., 2004). By analogy, UNC-51 and UNC-14 may regulate Rab5-mediated endocytosis to affect the regulation of UNC-5 localization. Unfortunately, we could not test this possibility, as rab-5(RNAi) causes embryonic death. Conversely, again by analogy, an attractive hypothesis is that UNC51.1 regulates the localization of the mammalian homolog of UNC-5 in neurons.

In C. elegans, autophagy is essential for dauer development and life-span extension (Melendez et al., 2003). Autophagy-related genes (unc-51/ATG1, unc-14/ATG6, M7.5/ATG7, lgg-1/ATG8 and F41E6.13/ATG18) are required for this process. In our RNAi analysis using rfp-3 mutants hypersensitive to RNAi, except for unc-51/ATG1, these autophagy-related genes were not required for DD/VD axon guidance or the localization of UNC-5. We think that conventional autophagy is not related to the localization of UNC-5, and that UNC-51/Atg1 has another unknown function in its localization.

UNC-51 and UNC-14 may be required for the maturation of UNC-5. Mutating the extracellular domain of GLP-1 (a Notch-like receptor) results in the accumulation of GLP-1 in the cell body (Wen and Greenwald, 1999). This accumulation is suppressed by a mutant of sel-9, whose product, SEL-9, is thought to function in the quality control of the endoplasmic reticulum-Golgi transport of GLP-1. In the unc-51 and unc-14 mutants, UNC-5 could accumulate in neural cell bodies because of a transport failure owing to quality control.

**UNC-5 may use a unique transport mechanism, silencing, for its surface localization at the growth cone**

In *Drosophila melanogaster*, the surface expression of Robo, a receptor for the repulsive guidance molecule Slit, is regulated by Comm (Georgiou and Tear, 2002; Keleman et al., 2002). If Comm and Robo are present in the same neuron, Comm sorts Robo into
vesicles bound for late endosomes and lysosomes (Keleman et al., 2005). If Robo is present without Comm, Robo is packaged into vesicles delivered to the growth cone. UNC-5 may also use a unique transport mechanism for its surface localization at the growth cone. In *C. elegans*, DD/VD neurons have ‘C’-shaped axons, of which the first anterior branch does not respond to Netrin/UNC-6 (Fig. 1B, Fig. 8B). This ‘silencing’ of UNC-5 function is probably not owing to the transcriptional regulation of unc-5, given that when unc-5 expression was under control of the unc-25 promoter, which drives expression from the birth of the DD/VD neurons into adulthood (Jin et al., 1999), the ‘C’-shaped axonal morphology was not affected (data not shown). Silencing of UNC-5 function has already been reported for the migration of hermaphrodite DTCs (distal tip cells) (Su et al., 2000); however, the molecular mechanism of this silencing is unknown.

We think that, throughout the extension of the first axonal branch, UNC-5 may be packaged in an UNC-51- and UNC-14-mediated manner into small vesicles, thus silencing the UNC-5 (Fig. 7B). Some unknown ‘UNC-5 surface signal’ may localize UNC-5 to the cell membrane at the right times and places for the growth cone to respond to Netrin/UNC-6 with dorsal extension. The regulation of UNC5SH1 (UNC-5 homolog) surface expression has been reported for mammalian cells (Williams et al., 2003).

Here we reported that the localization of the Netrin receptor UNC-5 was cooperatively regulated by UNC-51 and UNC-14. However, the molecular mechanisms underlying the localization of UNC-5 and the transport mechanism used are still unclear. The isolation and characterization of new mutants that have defects in UNC-5 and the transport mechanism used are still unclear. The potential and specific genetic interference by double-stranded RNA in Caenorhabditis elegans: *Nature* 391, 806-811.


