Caenorhabditis elegans *unc-51* gene required for axonal elongation encodes a novel serine/threonine kinase

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Mutations in the *unc-51* gene of the nematode *Caenorhabditis elegans* result in various abnormalities in axonal elongation and axonal structures. We cloned the *unc-51* gene by tagging with the transposon Tc1. The wild-type *unc-51* gene, which rescued the mutant phenotypes, encodes a novel serine/threonine kinase of 856 amino acids. Mutation sites were identified in the *unc-51* gene of six mutants. A Lys → Met mutation created in vitro in the kinase domain led to the loss of rescuing activity and was dominant negative, indicating that the kinase domain of Unc-51 is essential for the function. Expression of an *unc-51/lacZ* fusion gene was observed in many neurons at all stages. We propose that protein phosphorylation by the *unc-51* product is important for axonal elongation and possibly for axonal guidance.

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

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Construction of a complex neural network needs formation of axons and their guidance to the correct targets. Although many genes or proteins that are thought to be involved in this process have been identified (Dodd and Jessell 1988; Harrelson and Goodman 1988; Skene 1989; Hedgecock et al. 1990; Hall and Hedgecock 1991; McIntire et al. 1992; Li et al. 1992; Leung-Hagestain et al. 1992; Ishii et al. 1992; Goodman and Shatz 1993; Hamelin et al. 1993), the mechanism of axonal formation is still unclear. To analyze this mechanism, we have used the nematode *Caenorhabditis elegans*, because every neuron and the entire neuronal network of this animal have been identified by electron microscopy (White et al. 1986; Chalfie and White 1988) and because various molecular and genetic techniques are available.

The *unc* (uncoordinated movement)-51 gene of the nematode *C. elegans* was initially described by Brenner (1974). Although normal worms move actively on agar plates, *unc-51* mutants are mostly paralyzed, egg-laying defective, and dumpy. These behavioral defects are most probably related to defects in axonal formation, which has been observed in several specific neurons as described below. The reason for the dumpy phenotype is not known.

Six pairs of amphid neurons in the head and two pairs of phasmid neurons in the tail are thought to be chemosensory neurons in *C. elegans* (Hedgecock et al. 1985; Bargmann and Horvitz 1991). They take up 5-fluorescein isothiocyanate (FITC) and can be stained easily without fixation of the worms (Hedgecock et al. 1985).

The two pairs of phasmid neurons (PHA, PHB) can also be stained with the monoclonal antibody TY21 (Siddiqui 1989). In *unc-51* mutants, each of the posterior processes of these neurons was normal, but their anterior processes terminated prematurely at the ventral nerve cord, and the terminals tended to be enlarged (Hedgecock et al. 1985). The PDE neurons, which are likely to be mechanosensory neurons, do not normally take up FITC; but in certain mutants such as *cat-6* or *che-14* with abnormal mechanosensilla, these neurons sometimes can be stained with FITC (Hedgecock et al. 1985; Perkins et al. 1986). In *unc-51* mutants, the PDE neurons extended abnormal numbers of axons in abnormal directions, and these axons frequently had large varicosities close to the cell body (Hedgecock et al. 1985).

The HSN motor neurons control vulval muscles, and abnormalities of these neurons lead to the egg-laying defective phenotype (Desai et al. 1988). By use of an antibody specific to serotonin, these neurons were observed immunocytochemically (Desai et al. 1988; McIntire et al. 1992). In *unc-51* mutants, the HSN neurons entered the ventral nerve cord, but they failed to grow anteriorly along the ventral nerve cord. They also included ectopic, abnormally large varicosities near the cell body with extra neurites (Desai et al. 1988; McIntire et al. 1992).

The DD and VD neurons are thought to be inhibitory motor neurons in *C. elegans* (White et al. 1986; Chalfie and White 1988; McIntire et al. 1993a,b). These neurons are GABA-ergic and can be stained with antibody to GABA (McIntire et al. 1992, 1993a,b) or with the mono-

clonal antibody to β -tubulin (Siddiqui 1989, 1990). In *unc-51* mutants, the DD and VD axons are either prematurely terminated or are misdirected during circumferential elongation to the dorsal nerve cord (Siddiqui 1990; McIntire et al. 1992; see Fig. 2, below). In addition to these abnormalities, *unc-51* mutants have axonal defects in ALM, AVM, PLM, PVM, PHC, and PVN neurons (Siddiqui 1988, 1989, 1990; Siddiqui and Culotti 1991).

In *unc-51* mutants, axonal abnormalities have been observed in all the neurons examined to date; hence, this gene product plays an important role in construction of the neural network. In the present work, the *unc-51* gene was cloned by tagging with the transposon Tc1. We demonstrate here that the *unc-51* gene encodes a novel serine/threonine kinase whose activity is necessary for axonal elongation.

Results

Molecular cloning of the *unc-51* gene

To clone the *unc-51* gene in *C. elegans*, we screened progenies of a mutator strain RW7097 (Mori et al. 1988) for uncoordinated mutants. The mutator strain has a high rate of spontaneous mutation as a result of the insertion of transposons present at a high copy number, among which the Tc1 transposon is the most frequent. One isolate carried an *unc* mutation that was mapped to LG V. This mutation did not complement *unc-51(e369)* nor *unc-51(e1189)* mutations and reverted at a high frequency. These observations implied that this mutant had undergone insertion of a transposon in the *unc-51* gene. Therefore we cloned the *unc-51* gene with a Tc1 DNA probe. The DNA flanking Tc1 in the clone hybridized with a 4.3-kb *Eco*RI fragment in FK51 that was ob-

tained by repeated backcrosses of this mutant with the wild-type N2, whereas it hybridized with a 2.7-kb *Eco*RI fragment in N2 and four revertant strains isolated from the original *unc* mutant (data not shown). Because the size of Tc1 is 1.6 kb, the results suggest that these *Eco*RI fragments contain a part of the *unc-51* gene.

The cloned DNA rescued the *unc-51* mutant phenotypes

The 2.7-kb *Eco*RI fragment was physically mapped by A. Coulson (Coulson et al. 1986) on a cosmid F43A2. Initially, this cosmid was contained in a small isolated contig and was not localized precisely on the *C. elegans* genome map. It was mapped on chromosome V with *in situ* hybridization by D. Albertson (pers. comm.). Introduction of the DNA of cosmid F43A2 into the germ line rescued the uncoordinated, egg-laying defective, and dumpy phenotypes of *unc-51(e369)* to the wild type. A 13-kb *Eco*RI–*Sall* fragment of the cosmid (Fig. 1, subcloned in plasmid Bluescript, named pF14) also rescued completely. A 10-kb *Cla*I–*Sall* fragment within the above fragment that contains the complete coding region for the Unc-51 protein rescued, but only partially. Neither the left half (*Cla*I–*Apa*I) nor the right half (*Apa*I–*Sall*) of the 10-kb fragment rescued at all.

We then asked whether the defects of *unc-51(e369)* mutant in the axonal elongation could also be rescued by introduction of the 13-kb DNA fragment. When the DD and VD neurons of animals rescued to non-Unc phenotype were stained with anti-GABA antibodies (McIntire et al. 1992, 1993a,b), the axonal morphology was indistinguishable from that of the wild type (Fig. 2C). On the basis of the results of transposon tagging and rescue ex-

Figure 1. Genomic organization of *unc-51* gene and the results of phenotypic rescue experiments. The top line represents the 13-kb insert of pF14 that rescues the *unc-51* mutant phenotype. The exons in the *unc-51* gene are boxed: Solid boxes denote the kinase domain of the *unc-51* gene; stippled boxes denote the remaining translated region. SL1 is the splice-leader sequence (Huang et al. 1989); ATG denotes the putative initiation codon; and STOP shows the termination codon. [Poly(A)] stands for the poly(A) addition site. The mutation sites of *ks49*, *e369*, *ks38::Tc1*, *e432*, *e389*, and *e1120* are indicated. Lines below represent the inserts of plasmids used for the rescue of *unc-51(e369)* by germ-line transformation. (+) Complete rescue; (+/–) incomplete rescue; (–) no rescue. An asterisk (*) indicates a K → M mutation created *in vitro*. Abbreviations for the restriction sites: (C) *Cla*I; (E) *Eco*RI; (P) *Pst*I; (S) *Spe*I; and (A) *Apa*I.

Figure 2. Indirect immunofluorescence staining of GABA-ergic neurons (DD/VD) with antisera to GABA. (A) Wild-type animals; (B) *unc-51(e369)* mutant; (C) the *unc-51(e369)* rescued with the 13-kb *EcoRI-SalI* fragment of the wild-type *unc-51* gene shown in Fig. 1; (D) the *dpy-20(e2017)* mutant. (E) *dpy-20(e2017)* mutant that was transformed by coinjection of the *unc-51* (K → M) mutant gene and wild-type *dpy-20* gene. The top of the panel represents the dorsal side of the animal.

periments, we conclude that the 13-kb *EcoRI-SalI* fragment contains the functional *unc-51* gene.

Analysis of *unc-51* cDNA and mRNA

Roughly 1.5×10^5 plaques of a λ phage cDNA library, which was made from a *C. elegans* mixed-stage population of the wild-type N2, were screened with the *Tc1*-flanking fragment, and 10 positive clones were detected. The longest (BLO) had an insert of ~ 2.9 kb, excluding poly(A). This cDNA and most of the corresponding genomic regions were sequenced, which revealed that the *unc-51* gene includes nine exons (Figs. 1 and 3). To determine the 5' end of *unc-51* cDNA, we carried out PCR analysis in which we used a primer of the SL1 and SL2 splice-leader sequences found in *C. elegans* mRNAs (Huang and Hirsh 1989) and an antisense primer to the 5' end of BLO. We found that the SL1 existed 24 bp upstream of the 5' end of BLO (Figs. 1 and 3). The nucleotide sequence of the cDNA contained a single open reading frame that encodes a putative 856-amino-acid polypeptide, starting at the most 5' methionine codon of the open reading frame (Figs. 1 and 3). The AUG start codon is preceded by an in-frame terminator codon, thus, the entire protein coding sequence is covered. A poly(A) tail is present at the end of a 302-nucleotides long 3'-untranslated region, and a canonical polyadenylation signal (AATAAA) is located 17 bp upstream of the poly(A). These results indicate that the *unc-51* gene encodes a protein of 856 amino acids.

Putative *Unc-51* protein has a serine/threonine kinase catalytic domain near the amino terminus

The predicted amino acid sequence of the *Unc-51* protein comprises at least two distinct domains (Figs. 1 and 3). An amino-terminal part of the sequence (amino acids 14–275) shows a significant homology to eukaryotic protein kinases (Hanks et al. 1988). Most of the amino acid identities found between the conceptual *unc-51* translational product and protein kinases reside in highly conserved residues that are appropriately spaced in the 12 recognized subdomains of the catalytic domain (Hanks et al. 1988) (Fig. 4). The sequence motifs DLKPQN in subdomain VIb (residues 134–139) and GSPMYM in subdomain VIII (residues 180–185) of *Unc-51* indicate serine/threonine kinase substrate specificity (Hanks and Quinn 1991; Figs. 3 and 4).

By use of a data base that includes the sequences of ~ 500 kinases, the homology of the amino acid sequence of the predicted *Unc-51* kinase domain was examined by K. Kuma (Kyoto University, Japan). Highest homologies were found with SNF1-related protein kinase of *Arabidopsis thaliana* (38.1%, Le Guen et al. 1992) and with human p78 (37.2%, unpublished and found in GenBank 72 data base) (Fig. 4). Five other kinases in the data base showed an identity of $>32\%$, including products of the *Saccharomyces cerevisiae* SNF1 gene (Celenza and Carlson 1986), the *S. cerevisiae* *spk1* gene (Stern et al. 1991), and the *Schizosaccharomyces pombe* *nim1* gene (Feilott et al. 1991) (Fig. 4) and those of two other SNF1-related genes (data not shown). However, when a phylogenetic tree of these kinases and related kinases, including KIN I and KIN II of *S. cerevisiae* (Levin et al. 1987), rabbit skeletal myosin light chain kinase (Herring et al. 1990), and mouse calcium-calmodulin-dependent protein kinase IV (Jones et al. 1991), was constructed, *Unc-51* was found to be only distantly related to any of the other kinases analyzed (K. Kuma, pers. comm.). The relatively long carboxy-terminal portion (residues 276–856) of the putative *Unc-51* protein is distinct from all the other kinases so far identified and does not share significant sequence homologies with any of the proteins contained in the Swiss-Prot 28 (1994) data base. A hydropathy plot shows that the *Unc-51* product contains no apparent hydrophobic membrane-spanning domain similar to those found in a variety of integral membrane proteins (Kyte and Doolittle 1982). Thus, *Unc-51* seems to represent a novel kinase subfamily. It is similar to serine/threonine kinases SNF1, p78, KIN I, KIN II, and PoLo (Llamazares et al. 1991), in that they have a relatively long carboxy-terminal portion.

Mutation sites in the *unc-51* mutants

To determine functionally important regions of the *unc-51* gene, we localized mutation sites in six of the *unc-51* mutants (Figs. 1, 3; Table 1). In the *ks38::Tc1* allele, a *Tc1* was inserted within a protein-coding region downstream of the kinase domain. In the *e369* mutant, two point mutations were found: a silent mutation in the -1 opal

Figure 3. Nucleotide sequence of the cDNA and the deduced amino acid sequence (single-letter code). Protein-coding sequences are shown in uppercase letters and untranslated sequences in lowercase letters. The SL1 splice-leader sequence is indicated by a broken line. The catalytic domain of the *unc-51* protein kinase (Hanks et al. 1988) is underlined, and the presumed polyadenylation signal is indicated by double underlines. The stretches of amino acids suggesting serine/threonine substrate specificity (Hanks et al. 1988) are boxed. (V) The position of the Tc1 insertion in *ks38::Tc1*. The mutated codons in *ksEx9*, *e369*, *e389*, and *e1120* mutations are boxed. (V) The position of an intron is indicated by an arrowhead.

codon (TGA) to ochre (TAA), and an amber termination codon TAG in the CAG codon for Q553. The finding that *e369* has an amber (TAG) nonsense mutation is consistent with the previous result that this mutation was suppressed by tRNA^{Trp} amber suppressors *sup-5* and *sup-7* (Kondo et al. 1990). In the *e432* and *ks49* mutants, the 3' splice site G (5'-GT...AG-3') of the seventh and first introns, respectively, was mutated to A (5'-GT...AA-

3'). Abnormal transcripts, which used a cryptic 3' splice site (Aroian et al. 1993), were present in these mutants (Fig. 5A,B). In the *e389* mutant, a recombination or a complex rearrangement was found, resulting in frame-shift and premature termination (Fig. 5C). In the *e1120* mutant, a single G → A mutation was found near the carboxyl terminus of the putative coding region, changing the codon R841 (CGT) into H (CAT). We sequenced

Figure 4. Amino acid sequence comparison of the kinase domains of the Unc-51 product with those of other serine/threonine kinases. The amino acid sequence of the putative serine/threonine kinase catalytic domain of Unc-51 was aligned with SNF1-related protein kinase of *Arabidopsis thaliana* (Le Guen et al. 1992), p78 of human (unpublished and found in GenBank 72), SNF1 of *S. cerevisiae* (Celenza and Carlson 1986), Spk1 of *S. cerevisiae* (Stern et al. 1991) and Nim1 of *S. pombe* (Feilottter et al. 1991). Residues shared by all kinases are marked with asterisks (*); dots signify amino acids identical to Unc-51; dashes indicate space for alignment. The stretches of amino acids suggesting serine/threonine specificity of the Unc-51 protein kinase are underlined. The position of the amino acid at the beginning of the catalytic domain is indicated on the first line of each sequence. The percentage of amino acid identity is indicated in parenthesis.

the coding region of the *e1189* mutant, but no mutation site was found. The reduced *unc-51* mRNA level in this mutant (Fig. 6) suggests a mutation in a regulatory element outside of the coding region, which is important for transcription or mRNA stability.

Northern blot analysis for the *unc-51* alleles

To analyze the mRNA of the wild-type and mutant *unc-*

51 alleles, Northern blot analysis was done with the *unc-51* cDNA probe (Fig. 6). In the wild type, a 3.1-kb single band was seen (lane 1). In the *ks38::Tc1*, three bands were present (lane 3). We interpret the upper band to be an *unc-51* transcript containing *Tc1* sequence, and the middle band to be an intact or almost intact mRNA that arose from somatic excision of *Tc1* DNA (Moerman et al. 1988). The lower band presumably arose by trunca-

Table 1. The phenotypes of *unc-51* mutants and their mutation sites

Strains	Movement	Egg laying	Body shape	Mutation site (codon)
N2	active	normal	normal	
FK118(<i>e432</i>)	paralyzed	defective	dumpy (dpy)	3' splice site in intron 7, (GT ... AG) → (GT ... AA)
FK119(<i>e369</i>)	paralyzed	defective	dpy	-1 opal (TGA) → ochre (TAA) 553 Gln(CAT) → amber (TAG)
FK120(<i>e389</i>)	paralyzed	defective	dpy	801 Pro(CCC) → Thr(CTC) 809 Gln(CAG) ~823 Asn(AAT), deletions in 35 bp
FK121(<i>e1189</i>)	paralyzed	defective	dpy	no mutation found in the coding region
FK122(<i>e1120</i>)	paralyzed	defective	dpy	841 Arg(CGT) → His(CAT)
FK123(<i>ks49</i>)	somewhat active	defective	somewhat dpy	3' splice site in intron 1, (GT ... AG) → (GT ... AA)
FK51(<i>ks38::Tc1</i>)	fairly active	normal	somewhat dpy	<i>Tc1</i> insertion between 696 Asn(AAT) and 697 Thr(ACA)
FK124(<i>ksEx9</i>)	variable	variable	variable	39 Lys(AAG) → Met(ATG) (created in vitro)

The *unc-51* mutant strains of CB432(*e432*), CB369(*e369*), CB389(*e389*), CB1189(*e1189*), and CB1120(*e1120*) were backcrossed three times with N2. The resultant strains were named FK118(*e432*), FK119(*e369*), FK120(*e389*), FK121(*e1189*), and FK122(*e1120*). A mutant originally isolated by EMS treatment in this work was also backcrossed three times with N2, and FK123(*ks49*) was obtained. FK124(*ksEx9*) strain was obtained by introduction of an *unc-51* gene carrying a dominant negative mutation (Lys-39 → Met) into a *dpy-20* mutant together with *dpy-20* gene.

Figure 5. Mutations in the *e432*, *ks49*, and *e389* mutants. (A) In the CB432(*e432*) mutant, two PCR-amplified cDNA clones show the same sequence as N2 (Aroian et al. 1993) in this region, but the other two clones show a 12-bp deletion, probably generated by use of a cryptic 3' splice site as shown. (B) In the FK123(*ks49*) mutant, two PCR-amplified cDNA clones show 3-bp insertion but the other two clones show 7-bp deletions. These also arose by use of a cryptic 3' splice site as shown. (C) In the cDNA of the CB389(*e389*) mutant, a complex mutation(s) was found in the underlined region of 42-bp, resulting in the frameshift.

tion or initiation in the *Tc1* region. On the basis of the mRNA amounts relative to those of EF-1 α mRNA, *unc-51* mRNA levels in *e369*, *e389*, and *e1120* animals were not reduced much as compared with the wild type (lanes 2,4,6; see also the legend Fig. 6). It seems that these mutations did not affect the *unc-51* mRNA expression or stability. In *e432*, *ks49*, and *e1189* worms on the other hand, the relative mRNA levels were significantly reduced (lanes 5,7,8).

Expression of an *unc-51/lacZ* fusion gene

To analyze expression pattern of the *unc-51* gene, an *unc-51/lacZ* transcriptional fusion gene was constructed. The promoter region of the *unc-51* gene (the 4-kb *EcoRI*–*BstXI* fragment; see Fig. 1) was inserted into the pPD21.28 plasmid that carries the SV40 nuclear localization signal (Fire et al. 1990). This fusion construct was introduced into the germ line of the *unc-51(e369)* mutant together with the intact *unc-51* gene (the 13-kb *EcoRI*–*SalI* fragment in Fig. 1). Wild-type transformant lines were isolated and examined by staining with X-gal. In the stages from the first larval (L1) through the adult stage (Fig. 7A–E), the *lacZ* fusion gene was expressed strongly in many or most neurons including those in the

nerve ring near the head, those in the ventral nerve cords, and those near the tail. The *lacZ* fusion was also expressed in some non-neuronal cells, probably in body wall muscle nuclei (Fig. 7C,E) and pharyngeal muscle nuclei (Fig. 7E) (Okkema et al. 1993). The expression pattern was similar in these stages. Extensive and rather uniform staining was observed in the embryos (Fig. 7F).

As an approach to study the intracellular localization of the Unc-51 protein, an *unc-51/lacZ* translational fusion gene without a nuclear localization signal was constructed by insertion of the *lacZ* gene into the 13-kb *EcoRI*–*SalI* fragment at the *XhoI* site shown in Figure 1 (after E289 in the amino acid sequence). The expression pattern of this construct was similar to that of the previous promoter fusion with a nuclear localization signal, except that staining was observed in the ventral nerve cord as well as in cell bodies (Fig. 8).

Dominant-negative effect of a mutation in the kinase domain

A homology search of the putative Unc-51 protein suggested that Unc-51 has serine/threonine kinase activity.

Figure 6. Northern blot analysis of poly(A)⁺ RNA of wild-type and *unc-51* mutant worms. Poly(A)⁺ RNA (5 μ g) was resolved and blotted to nylon filter, and probed with *unc-51* cDNA. (Lane 1) N2; (lane 2) *e369*; (lane 3) *ks38::Tc1*; (lane 4) *e389*; (lane 5) *e432*; (lane 6) *e1120*; (lane 7) *ks49*; (lane 8) *e1189*. The size of the RNA was estimated in reference to the 23S and 18S rRNAs. The same filter was rehybridized with the cDNA of *C. elegans* elongation factor 1 α homolog (M. Koga, pers. comm.). *unc-51* mRNA levels normalized by EF-1 α mRNA levels were 100% (N2), 67% (*e369*), 181%, 80%, and 141% for *top*, *middle*, and *bottom* bands [*ks38::Tc1*], 76% (*e389*), 36% (*e432*), 96% (*e1120*), 29% (*ks49*), and 32% (*e1189*).

Figure 7. Expression pattern of an *unc-51/lacZ* promoter fusion gene with a nuclear localization signal. The *unc-51(e369)* mutant was transformed to non-Unc phenotype by germ-line introduction of the intact *unc-51* gene and the plasmid pF4.0LZ. Animals were fixed and stained with X-GAL. (A) L1 (285 $\times$); (B) L2 (285 $\times$); (C) L3 (285 $\times$); (D) young adult (71 $\times$); (E) head region of the young adult (285 $\times$); and (F) late embryos (285 $\times$).

However, no mutation was found in the kinase domain. To analyze the importance of the kinase activity, the AAG codon of the genomic DNA corresponding to lysine (39) in the kinase domain was mutagenized to ATG for methionine in vitro. This lysine (39) is thought to be in an ATP-binding site of kinase domain (Han et al. 1993) and perfectly conserved in all protein kinases (Hanks et al. 1988); therefore, the mutagenized protein should not have any kinase activity. As expected, this mutagenized *unc-51* gene did not rescue the mutant phenotypes of *unc-51(e369)* (Fig. 1).

To further confirm the importance of the Unc-51 kinase activity, a possible dominant-negative effect of the mutagenized *unc-51* gene was analyzed as described by Han et al. (1993). In *C. elegans*, extrachromosomally

transformed animals have several hundred copies of introduced genes, and the gene products are likely to be hyperproduced. If the kinase activity is essential for axonal elongation, inhibition of the functional Unc-51 kinase by a competition for the substrate or a possible regulatory factor with the hyperproduced Unc-51 mutant proteins should lead to the mutant phenotypes. The Lys (39) to Met *unc-51* mutant gene (pF14M) was introduced into *dpy-20(e2017)* worms with the intact *dpy-20* gene (pMH86) as a marker at the ratio of 9:1. Most of the transformed animals showed phenotypes characteristic to *unc-51* mutants (Unc, Dpy, egg-laying defective, and axonal elongation defects) (Fig. 2E). Therefore, the kinase activity of the Unc-51 protein is probably essential for the function. The wild-type gene (pF14) was also intro-

Figure 8. Expression of an *unc-51/lacZ* translational fusion gene without a nuclear localization signal. The plasmid pF14LZ was introduced into the *unc-51(e369)* mutant together with the intact *unc-51* gene. Non-Unc transformants were fixed and stained with the anti-LacZ antibody. (A) 552 $\times$; (B) 875 $\times$.

duced into *dpy-21(e2017)* worms with the intact *dpy-20* gene, but no stable transformant lines were obtained. It is likely that overexpression of the wild-type gene is toxic, and therefore transformants are lethal.

Discussion

The unc-51 gene encodes a novel serine/threonine kinase

Sequence analysis of the *unc-51* cDNA showed that the predicted Unc-51 protein is composed of at least two distinct domains, a serine/threonine kinase catalytic domain (Hanks et al. 1988) near the amino-terminal and a relatively long carboxy-terminal domain with an unknown function. In a phylogenetic analysis, the kinase domain could not easily be classified into any of the known serine/threonine kinase subfamilies (Hanks et al. 1988), and the unusually long carboxy-terminal portion did not share significant homologies with any of the proteins so far identified. It seems likely that the Unc-51 protein is a novel serine/threonine kinase.

Analysis of *unc-51* mutant alleles

Mutant analysis showed that *e369*, *ks38::Tc1*, *e432*, *e389*, and *e1120* had mutations in the carboxy-terminal portion and *e1189* had no mutation site in its coding

region (Figs. 1,3; Table 1). Therefore, their Unc-51 products may have kinase activities if they are produced. Three mutants *e432*, *ks49*, and *e1189*, showed significantly decreased *unc-51* mRNA levels (Fig. 6). Both *e432* and *ks49* mutants have a mutation at the 3' splice site of an intron; hence such mutations decrease the efficiency of pre-mRNA splicing. *FK51(ks38::Tc1)* mutant is fairly active as compared with other mutants, and it is not egg-laying defective. Northern blot analysis showed evidence of intact *unc-51* mRNA in addition to abnormal mRNAs (Fig. 6, lane 3). In this mutant, the *Tc1* may be excised somatically (Moerman et al. 1988) or a part of the *Tc1* sequence may be spliced out during mRNA formation, and some active Unc-51 proteins are probably synthesized.

The mutation in the kinase domain created *in vitro* revealed that the kinase domain of Unc-51 is essential for the *in vivo* function. Such a mutation in the kinase domain is probably null. It is remarkable that all the mutation sites found in the *unc-51*-coding sequences of the isolated mutants were downstream of the kinase domain. In *e369*, *e389*, and *e1120* mutants, their *unc-51* mRNA levels were not reduced as much as the wild type (Fig. 6). These results suggest that the carboxy-terminal portion is also important for the function of Unc-51 protein. Mutations *e369*, *e389*, and *e1120* may possibly be null based on their phenotypes. Alternatively, null mutations might be lethal. Mutations *ks49* and *ks38::Tc1* are apparently reduced function mutations.

Expression of the *unc-51* gene

During embryonic development when many neurons are extending their axons, the *unc-51/lacZ* fusion gene shows extensive expression, particularly in the head region of late embryos (Fig. 7F). In the larval stages, expression of the *lacZ* fusion appears to be restricted to neurons (Fig. 7A–C). Therefore, Unc-51 protein is likely to be necessary in neurons rather than in the cells along which their axons run. Furthermore, many neurons clearly expressed the *unc-51/lacZ* fusion gene in adult worms in which the axonal network had been constructed (Fig. 7D,E). This finding suggests that the Unc-51 protein is required not only for axonal elongation but also either for the maintenance of axons by, for example, participating in membrane turnover (see the next section), or for an unknown neuronal function. Because most neurons expressed this fusion gene, we speculate that *unc-51* mutants have axonal defects in the unexamined neurons as well as in DD, VD, PDE, and HSN neurons.

The function of Unc-51 protein in the formation of normal axonal structures

Expression pattern of the *unc-51/lacZ* fusion gene and observations of axonal defects in *unc-51* mutants suggest that Unc-51 protein is required for axonal formation or elongation of most neurons. In support of this view, McIntire et al. (1992) found electron microscopic abnor-

malities of axons such as enlargement of the diameter, atypical membranous vesicles, and cisternae-like structures in the axons of *unc-51* mutants. They proposed that the outgrowth defects in *unc-51* mutants could perhaps be a consequence of abnormalities in the structural components of axons, rather than in the components directly involved in guiding axonal elongation. Atypical membranous vesicles and cisternae-like structures are also found within presynaptic terminals in the temperature-sensitive *shibire* mutant of *Drosophila* (Koenig et al. 1983; Kosaka and Ikeda 1983). Blockage of neurite development and growth cone formation was found in neuronal cultures from the *shibire* mutant at the restrictive temperature, and these defects were presumed to be related to defective membrane recycling and endocytosis (Kim and Wu 1987). The *shibire* gene encodes a protein related to rat dynamin (van der Bliek and Meyerowitz 1991; Chen et al. 1991), a mechanochemical enzyme that interacts with microtubules (Obar et al. 1990; Vallee and Bloom 1991). Recently, it was reported that dynamin is identical to the neuronal phosphoprotein dephosphin (p96) and that on excitation, dynamin is phosphorylated and is quantitatively dephosphorylated in the nerve terminal (Robinson et al. 1993). Because the Unc-51 protein is localized in axons and neural cell bodies (Fig. 8), it is tempting to speculate that the Unc-51 protein is involved in the membrane recycling of the axons by phosphorylation of a *C. elegans* dynamin homolog or another component in the pathway that includes a dynamin homolog.

Another possibility is that the Unc-51 protein phosphorylates and regulates a component that is not related to dynamin function but is required for the formation or function of the neuronal axons. Among such components in mammals, several proteins are known to be phosphorylated, such as GAP-43 (Skene 1989), calpactin I and II, synaptophysin, and synapsins (Trimble et al. 1991). Among these, at least synapsins are phosphorylated at serine residues. In *C. elegans*, *unc-14*, *unc-33*, and *unc-44* mutants have axonal abnormalities similar to those in *unc-51* mutants (Hedgecock et al. 1985; McIntire et al. 1992). Therefore, the function of the *unc-51* gene might be related to the functions of the genes specified by these mutants, or to the functions of *C. elegans* homologs of the phosphorylated components in mammals.

Possible functions of Unc-51 protein in axonal guidance or fasciculation

In *unc-51* mutants, axonal guidance and fasciculation seem to be abnormal in addition to various abnormalities in axonal structures. This apparent defect in axonal guidance and fasciculation may be solely due to a defect in the structure or function of the axonal membrane. Alternatively, it is possible that Unc-51 regulates the function or synthesis of a component(s) more directly involved in guidance or fasciculation, in addition to its function in the axonal structures. For example, in *unc-51* mutants, the DD and VD axons terminate during cir-

cumferential elongation to the dorsal nerve cord, and are abnormally defasciculated in the ventral nerve cord (Siddiqui 1990; McIntire et al. 1992; Fig. 2). The dorsal elongational defects of DD and VD axons were also observed in *unc-5* and *unc-6* mutants of *C. elegans* (Hedgecock et al. 1990; Siddiqui 1990; McIntire et al. 1992). The two genes encode a putative adhesion molecule (Leung-Hageteijn et al. 1992) and a novel laminin-related molecule (Ishii et al. 1992), respectively. Hedgecock et al. (1990) proposed that the Unc-6 proteins build up a circumferential gradient along the epidermal surface of *C. elegans*, and that the Unc-5 proteins function as the receptor at dorsal migrations (Hamelin et al. 1993). If this is the case, it is possible that in the DD/VD neurons, Unc-51 protein functions by the phosphorylation of a molecule in the pathway that transmits the Unc-6 gradient signal through the Unc-5 receptor. The defasciculations of the DD and VD axons of the ventral nerve cord found in the *unc-51* mutant may occur as a result of the inactivation or loss of some adhesion molecules like fasciclins of *Drosophila* (Grenningloh et al. 1991; Elkins et al. 1990). The expression of *unc-51/lacZ* translational fusion product in the ventral nerve cord (Fig. 8) supports this idea. The Unc-51 protein kinase might regulate such activities by phosphorylation.

Materials and methods

C. elegans strains

The *unc-51* mutant strains CB369(e369) and CB1189(e1189) were provided by the *Caenorhabditis* Genetics Center (USA). CB389(e389), CB1120(e1120) and CB432(e432) were provided by the Laboratory of Molecular Biology, Medical Research Council (MRC) (UK). Worms were grown on NGM agar plates at 20°C fed with the OP50 strain of *Escherichia coli* (Wood 1988).

Cloning and structural analysis of the *unc-51* gene

Molecular manipulations were performed essentially according to Sambrook et al. (1989).

Molecular cloning of the 4.3-kb fragment in the *unc-51* gene was performed as follows. The total DNA of FK51 was digested with *EcoRI*, and size-fractionated by electrophoresis in a 0.8% agarose gel. The 4- to 5-kb fragments were purified and ligated to pUC19 vector. The resultant *C. elegans* partial genomic library was screened with the Tc1 DNA probe. From each of the positive clones, Tc1 was excised with *EcoRV* and the flanking sequence was used as a probe for Southern hybridization for the RFLP analysis. Hybridization was performed in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 µg/ml of salmon sperm DNA, and 50% formamide at 42°C for 20 hr. The DNA probe was labeled with random priming of the DNA clone. Final washing was in 2× SSC, 0.1% SDS, at 37°C. Autoradiography was done at -70°C for 40 hr with an intensifying screen.

The wild-type *unc-51* cDNA clone pBLO was isolated from a λ Zap library (Barstead and Waterston 1989). Bluescript SK(-) plasmids carrying the cDNA were excised in vivo following the protocol of Stratagene. The cDNA sequences were obtained by sequencing sets of nested exonuclease III deletions (Henikoff 1984) as well as various subcloned fragments in M13 or pUC18. Sequencing was carried out with the dideoxy chain termination method in both directions by use of Sequenase (U.S. Biochem-

ical). The 5' end of the wild-type *unc-51* mRNA was determined by PCR analysis with the SL1 splice-leader sequence primer (5'-GGTTTAATTACCCAAGTTTGAG-3') and an antisense primer (5'-AAGCCGTCAAAGTCTCCAT-3') near the 5' end of pBLO.

The mutation sites were located by sequencing reverse transcription-PCR products (Sambrook et al. 1989) of the mutant poly(A)⁺ RNA, except for *ks38::Tc1* in which the Tc1 flanking region was sequenced. In *e432*, PCR-amplified genomic DNA containing the 7th intron was sequenced, because abnormal splicing was observed.

Transformation of *C. elegans*

Following the method of Mello et al. (1991), DNA carrying a genomic fragment was injected into the syncytial gonads of *unc-51(e369)* adult hermaphrodites at a total concentration of 100 ng/μl. The plasmid pTRU-1, which contains an *unc-22* antisense RNA expression gene derived from pPD10.46 (Fire et al. 1991) and *rol-6(su1006)* gene (Kramer et al. 1990) derived from pRF4 was co-injected at 1:1 DNA ratio as a selection marker, and worms with the *unc-22* mutant phenotype (twitcher) were selected. For cosmid F43A2 or pF14 carrying the 13-kb *EcoRI*-*SalI* fragment, rescue of the *unc-51(e369)* phenotype was confirmed by microinjection of the DNA without another selection marker, but with 10-fold excess of a vector plasmid Bluescript SK(+) for dilution, because injection of cosmid F43A2 alone could not rescue the mutant phenotypes and therefore high copy numbers of this cosmid may be toxic. In this case, animals with the wild-type (non-Unc) morphology were selected as transformants.

RNA procedures

Total RNA from a mixed population of wild type (strain N2) and *unc-51* mutant worms was prepared by the guanidine isothiocyanate method and poly(A) RNA was enriched on an oligo(dT)-cellulose column (Sambrook et al. 1989). Five micrograms of the poly(A) RNA of the worms was resolved on a 1% formaldehyde-agarose gel and blotted onto a nylon membrane filter (Biodyne A). Hybridization was performed in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml of salmon sperm DNA, and 50% formamide at 42°C for 20 hr. The probe was labeled with random priming of the 0.9-kb *EcoRI* fragment of the *unc-51* cDNA clone. Final washing was done in 2× SSC, 0.1% SDS, at 20°C. Autoradiography was done with an imaging plate for 24 hr, which was analyzed with Fuji image analyzer BAS2000 (Fuji film). The filter was used for rehybridization with the *C. elegans* EF-1 α probe (M. Koga, pers. comm.).

Indirect immunofluorescence for GABA antibody and LacZ antibody

Staining with anti-GABA antibody was performed essentially according to the protocol described by McIntire et al. (1992) and Link et al. (1992). Staining with LacZ antibody was performed essentially according to the protocol described by Finney and Ruvkun (1990) except for the protocol of fixation (2% paraformaldehyde and 1× MRWB for 45 min at 4°C). LacZ antibody was obtained from Cappel. The specimens were observed by fluorescence microscopy (ZEISS Axioplan).

Construction and expression of lacZ fusion constructs

The putative promoter region of the *unc-51* gene (the 4-kb *EcoRI*-*BstXI* fragment) was subcloned into the *SmaI* site of the

lacZ expression vector pPD21.28 (Fire et al. 1990). This plasmid was named pF4.0LZ. A mixture of pBluescript SK(+) (for dilution), pF14, and pF4.0 LZ at the ratio of 10:1:1 was used for the transformation of the *unc-51(e369)* mutant. Fixation and staining was performed by the protocol described by Fire et al. (1990). The *lacZ* gene was subcloned in frame into the pF14 plasmid at the *XhoI* site. This plasmid was named pF14LZ. A mixture of pBluescript SK(+) (for dilution), pF14, and pF14LZ at the ratio of 10:1:1 was used for transformation of *unc-51(e369)* mutants. Animals of a non-Unc transformant line was used for staining with LacZ antibody.

Preparation and analysis of a mutation in the kinase domain

In vitro mutagenesis was done essentially according to the Kunkel method (Sambrook et al. 1989). The 1-kb *HindIII*-*EcoRI* genomic DNA fragment including the Lys codon at position 39 and the 17-mer antisense mutagenized oligonucleotide (K → M) 5'-CGATCGCCATGATGGCA-3' were used for mutagenesis. This mutagenized fragment was used for reconstruction of the entire 13-kb DNA fragment corresponding to the *unc-51* gene. The pF14M plasmid DNA bearing the mutagenized *unc-51* gene was injected into *dpy-20* worms together with pMH86 plasmid that contains the intact *dpy-20* gene as a marker. The concentration of each plasmid used for injection was 90 ng/μl with pF14M and 10 ng/μl with pMH86 in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Transformants were selected and analyzed for their axons by indirect immunofluorescence of GABA.

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