The conserved kinase UNC-51 acts with VAB-8 and UNC-14 to regulate axon outgrowth in *C. elegans*

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Summary

Directional cues guide growth cones. While molecules like UNC-6/netrin direct migrations along the dorsoventral axis of many organisms, it is unclear how anteroposterior guidance is achieved. We describe a physical interaction between VAB-8, a protein both necessary and sufficient for posteriorly directed migrations in *C. elegans*, and UNC-51, a conserved serine/threonine kinase that functions generally in axon outgrowth. We show that both proteins function in the CAN neurons to direct their axons posteriorly. Expression in the CANs of peptides predicted to interfere with interactions between UNC-51 and both VAB-8 and UNC-14, a second protein that interacts physically with UNC-51, disrupts CAN axon outgrowth. We provide genetic evidence that VAB-8 functions in an UNC-51 pathway for posteriorly directed CAN axon guidance and show that VAB-8 and UNC-14 can be targets of UNC-51 kinase activity. Taken together, our results suggest that VAB-8 and UNC-14 are substrates that mediate the function of UNC-51 in axon outgrowth.

Key words: *C. elegans*, UNC-14, UNC-51, VAB-8, Axon guidance

Introduction

The migrations of neurons and their growth cones contribute to the final form and pattern of connectivity of all nervous systems. How does a cell or axon growth cone navigate through a complex cellular environment to reach its correct destination? One general theme that has emerged over the last decade is that guidance molecules attract or repel neurons or axon growth cones to regulate their migrations. An active area of research in developmental neurobiology has been the identification of these guidance molecules and their receptors. One of the first guidance cues identified was the *C. elegans* protein UNC-6, a secreted molecule that is necessary for the directed migrations of cells and axon growth cones along the dorsoventral axis (Hedgecock et al., 1990; Ishii et al., 1992; McIntire et al., 1992; Wadsworth et al., 1996). Homologs of UNC-6, known as netrins, were subsequently shown to guide growth cones along the dorsoventral axis of the vertebrate spinal cord (Kennedy et al., 1994; Serafini et al., 1994). The receptors for UNC-6/netrin, UNC-5 and UNC-40, are also conserved and function in dorsoventral guidance in nematodes, flies and vertebrates (Chan et al., 1996; Chisholm and Tessier-Lavigne, 1999; Keino-Masu et al., 1996; Keleman and Dickson, 2001; Kolodziej et al., 1996; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992).

While UNC-6/netrin and its receptors guide cells and growth cones along the dorsoventral axis in many organisms, an equivalent global guidance system for anteroposterior (AP) migrations has not been identified. Several molecules involved in AP guidance, however, have been described. An AP gradient of Wnt4 was recently shown to guide commissural axons anteriorly along the rat spinal cord (Lyuksyutova et al., 2003). In *C. elegans*, two molecules have been implicated in AP guidance. The anteriorly directed migrations of the right Q neuroblast and its descendants require MIG-13, a conserved cell surface molecule. While loss of MIG-13 is specific in its effects, expression of *mig-13* from a heat-shock promoter shifts the final positions of many migratory cells anteriorly, whether they normally migrate anteriorly or posteriorly (Sym et al., 1999). *C. elegans* VAB-8, by contrast, is both necessary and sufficient for posteriorly directed migrations (Wightman et al., 1996; Wolf et al., 1998). *vab-8* encodes two novel intracellular proteins, VAB-8L and VAB-8S (Wolf et al., 1998). VAB-8L is 1066 amino acids long and contains an N-terminal kinesin-like motor domain and a novel C terminus. VAB-8S lacks the kinesin-like motor domain. VAB-8L functions in all *vab-8*-dependent axon migrations, as well as in some cell migrations. VAB-8S functions in a subset of *vab-8*-dependent cell migrations. Both forms of VAB-8 can function cell autonomously in cell migration and axon guidance (Wolf et al., 1998). Based on the sequence of its motor domain, VAB-8 has been placed in a subfamily of divergent kinesin-like molecules (Miki et al., 2001).

How does *vab-8* carry out its function in directing posterior migrations? To identify proteins that interact with VAB-8, we conducted a yeast two-hybrid screen using VAB-8L as bait and identified UNC-51. *unc-51* mutants exhibit axon outgrowth defects, though the defects are not restricted to posteriorly directed axons (Hedgecock et al., 1985; McIntire et al., 1992). *unc-51* mutant axons also have unusually large varicosities, and electron microscopy revealed abnormal vesicles and cisternae-like structures within the axons (McIntire et al., 1992).

The gene *unc-51* encodes a serine/threonine kinase that is expressed in all *C. elegans* neurons (Ogura et al., 1994). Mouse homologs of UNC-51, Unc51.1 and Unc51.2, are expressed in the developing and mature cerebellum, as well as in cultured granule cells (Tomoda et al., 1999). Transfection of a dominant negative form of Unc51.1 in cultured granule cells inhibited neurite formation and extension, suggesting that the function of *unc-51* in neurite outgrowth is conserved (Tomoda et al., 1999).

UNC-51 can also bind to UNC-14, a novel *C. elegans* protein involved in axon outgrowth (Ogura et al., 1997). *unc-51* and *unc-14* mutants display similar axon defects, although the phenotypes of *unc-14* mutants are less severe than those of *unc-51* mutants (McIntire et al., 1992). Like *unc-51, unc-14* is expressed in most neurons (Ogura et al., 1997).

In this study, we describe the physical interactions between VAB-8 and UNC-51, and provide genetic evidence that the interactions of UNC-51 with VAB-8 and UNC-14 are necessary for posteriorly directed axon outgrowth. We also show that VAB-8 and UNC-14 can be substrates for UNC-51 kinase activity. We propose that UNC-51 regulates VAB-8 and UNC-14 to direct axon outgrowth posteriorly.

Materials and methods

Yeast two-hybrid screening and binding assays

A full-length *vab-8* cDNA was amplified by PCR from pV8SL (Wolf et al., 1998) and subcloned into the pAS2 vector (Clontech). We fused VAB-8L to the N-terminal GAL4 DNA-binding domain to construct the bait plasmid. A mixed-staged *C. elegans* cDNA library provided by Robert Barstead was amplified with random-primed oligonucleotides. The methods and protocols for the yeast two-hybrid screen were adapted from Durfee et al. (Durfee et al., 1993), Aspenstrom and Olson (Aspenstrom and Olson, 1995), and protocols from MATCHMARKER Two-Hybrid System 2 (Clontech). Y190 containing the yeast two-hybrid vectors was grown at 30°C on synthetic dextrose minimal medium (SD) containing 2% glucose plus the required amino acids.

About 500,000 clones were screened using the full-length VAB-8 as bait. 46 clones were positive for both growth on histidine and β-galactosidase activity. Only three out of the 46 clones required the presence of the library prey plasmids to activate the GAL4 promoter.

For the yeast two-hybrid binding assay, sequences of various domains of UNC-51 were generated by PCR using pBLO (vector containing *unc-51* cDNA) (described by Ogura et al., 1994) as template. The resulting PCR products were subcloned into the pACTII vector (Clontech).

In vitro binding

Sequences of various domains of VAB-8 were generated by PCR using pV8SL and subcloned into pCITE-4a(+) vector (Novagen). *unc-14* sequences were amplified by PCR from pR4BK1, a plasmid containing the full-length *unc-14* cDNA (Ogura et al., 1997) and subcloned into the pCITE-4a(+) vector. *unc-51* sequences were amplified by PCR from pBLO and subcloned into pGEX-4T-2 vector to generate GST fusion proteins (Pharmacia Biotech).

GST fusion proteins were expressed in *E. coli*, strain BL21(pLys$^-$) (Studier et al., 1990), and purified and bound to glutathione-Sepaharose 4B beads according to the manufacturer's protocol (Pharmacia Biotech). The various bound fusion proteins were quantified by comparison to known proteins on SDS-PAGE gels stained with GelCode Blue Stain Reagent (PIERCE). 10-20 μg of bead-conjugated GST fusion proteins were exchanged into binding buffer [20 mM Hepes, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.2% Triton X-100, 1% BSA, protease inhibitors (Calbiochem, 539134), pH 7.6] and rocked for at least 30 minutes at 4°C before addition of in vitro transcribed and translated protein fragments. In vitro transcription and translation of VAB-8 protein fragments were carried out using the TnT Quick Reticulocyte Lysate System (Promega, L1170). To the lysate system, 1 μg of pCITE-VAB-8 plasmid was added as transcription template, and 20 μCi of [^{35}S]methionine (>1000 Ci/mmole) was added to label the protein products. Freshly synthesized protein fragments were added to bead-bound GST fusion proteins and allowed to bind overnight at 4°C. The beads were then washed four times in wash buffer (10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) and resuspended in 2× sample buffer (125 mM Tris pH 6.9, 20% glycerol, 4.2% SDS, 3% 2-mercaptoethanol, 1% bromophenol blue). Proteins were denatured at 85-90°C for 10 minutes and separated on 12% or 18% SDS-PAGE gels. The gels were dried onto Whatman paper and exposed to film (Kodak Biomax MR) overnight.

C. elegans strains

Strains were maintained at 20°C as described by Brenner (Brenner, 1974). The mutations used in this work were: *unc-14(e866)*I (Ogura et al., 1997), *vab-8(ev411)*V (Wightman et al., 1996; Wolf et al., 1998), *unc-51(e369)*V (Brenner, 1974; Ogura et al., 1994), and *unc-51(e1120)*V (Ogura et al., 1994).

The promoter::gene fusion DNA constructs were injected into adult hermaphrodites and maintained as extrachromosomal arrays (Mello et al., 1991).

ceh-23 promoter constructs

GFP is expressed in the CAN cells of animals bearing the *Pceh-23::gfp* transgene (pTF1). pTF1 was constructed by cutting a 7 kb *ceh-23* promoter region with *Sph*I and *Sma*I and subcloning the fragment into pPD95.77 (Fire lab 1995 vector kit). A solution containing 50 ng/μl of pTF1 and 50 ng/μl of pRF4 [*rol-6(su1006)*] was injected into the wild type to produce the extrachromosomal array *gmEx217*.

All the other *ceh-23* promoter constructs were generated by PCR amplification and by subcloning into the *Sma*I site of pTF1.

Cell autonomy experiments

A solution containing 1 ng/μl pceh-23::vab-8L::gfp and 100 ng/μl pTF1 was injected into *vab-8 (ev411)* to produce the extrachromosomal array *gmEx294*. A solution containing 1 ng/μl pceh-23::unc-51::gfp and 100 ng/μl pTF1 was injected into *unc-51(e369)* to generate the extrachromosomal array *gmEx278*.

Peptide expression experiments

pceh-23::vab-8(332-514)::gfp contains the cDNA sequences of VAB-8 that encode amino acids 332-514. These sequences were subcloned into pTF1 as described above. A solution containing 50 ng/μl pceh-23::vab-8(332-514)::gfp, 50 ng/μl pTF1 and 10 ng/μl pmyo-2::gfp was injected into wild-type hermaphrodites to generate the extrachromosomal array *gmEx266*. pceh-23::unc-51(451-856)::gfp contains sequences corresponding to amino acids 451 to the C-terminal end (aa 856) of UNC-51. A solution containing 100 ng/μl pceh-23::unc-51(451-856)::gfp, 50 ng/μl pTF1 and 20 ng/μl pflp-1::gfp (a gift from Chris Li) was injected into wild type to generate extrachromosomal array *gmEx264*.

vab-8 overexpression

To overexpress *vab-8*, a solution containing 100 ng/μl pFWV8LG, 100 ng/μl pTF1 and pRF4 [*rol-6(su1006)*] DNA was injected into wild type to generate the extrachromosomal array *gmEx230*. pFWV8LG is a gene-cDNA fusion of *vab-8* that expresses the VAB-8 long form from the *vab-8* promoter (Wolf et al., 1998).

CAN axon scoring

To score CAN axons, L4 hermaphrodites were immobilized in 5% sodium azide and viewed under the 40× objective using a reticule

(Zeiss). Measurements were taken of: the distance between the CAN cell body and the center of the vulva; the distance between the center of the vulva and the PHA/B neurons; and the length of the CAN's posterior axon. Percentage of migration was determined by dividing the CAN posterior axon length by the distance between the CAN cell body and the PHA/B neurons. CAN cell bodies were frequently misplaced anteriorly in many of the strains. To ensure that this defect did not affect the scoring of axon length, only axons whose cell bodies were located within 40 μm of the center of the vulva were scored as data points. The axons were scored as having a misrouting defect if they had turned away from their normal trajectory and migrated in the wrong direction.

Posterior axons extending 95-100% of the distance to the PHA/B neurons were considered wild type and were grouped together in the most posterior box of the scoring charts. We used a two-tailed Z test to determine if the percentages of wild-type axons were statistically different between two strains (two sample proportions, WebStat 3.0 program on http://www.webstatsoftware.com).

COS cell transfection and sample preparation

PCR-generated full-length *vab-8* cDNA sequences were subcloned into vector pcDNA/myc-HisB (Invitrogen) to generate pcDNA-vab-8-myc. PCR-generated sequences of full-length *unc-51* cDNA plus an extra C-terminal FLAG tag were subcloned into vector pFLAG-CMV-5a (Sigma) to generate pCMV-unc-51-FLAG. PCR-generated sequences of full-length *unc-14* cDNA plus a C-terminal HA tag were subcloned into vector pcDNA3 (Invitrogen) to generate pcDNA-unc-14-HA.

COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum at 37°C. Transfections were carried out using the LipofectAminePlus Reagent (Invitrogen, 10964-013). For each 10-cm diameter dish, 5 μg of pcDNA-vab-8-myc, 5 μg pcDNA-unc-14-HA, 3 μg pCMV-unc-51-FLAG, 3 μg pCMV-unc-51(K39R)-FLAG, or 3-6 μg pCMV-unc-51(ΔAIKAI)-FLAG was used. Cells were harvested about 24 hours after transfection. Cells were detached from dishes by treatment with trypsin, washed with 1× PBS, resuspended in 0.7-1 ml cold 1% Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris, 300 mM NaCl, 5 mM EDTA, protease inhibitors, pH 8.0 at 4°C), and allowed to lyse on ice for 15 minutes.

To immunoprecipitate (IP) the UNC-51-FLAG fusion protein, 2.5-3 μl of anti-FLAG M2 monoclonal antibody (Stratagene, 200472) was added per ml of pre-cleared cell extract. To IP the VAB-8-Myc fusion protein, 12 μl of monoclonal anti-Myc antibody (Covance, MMS-150R) was added. To IP the UNC-14-HA fusion protein, 12 μl of monoclonal anti-HA antibody (Covance, MMS-101R) was added. Samples were nutated at 4°C for 2 hours to allow antibody binding, and then 40-120 μl resuspended protein G-Sepharose beads (Pharmacia Biotech) were added to capture the antibody-protein complex. The mixture was nutated for 30 minutes at 4°C. The beads were washed with 0.1% Triton wash buffer (0.1% Triton X-100, 50 mM Tris, 300 mM NaCl, 5 mM EDTA, pH 8.0 at 4°C) and exchanged into appropriate buffers for the subsequent reactions.

In vitro phosphatase and kinase reactions

IP bead samples were washed with phosphatase (PPase) wash buffer (50 mM Tris, 0.1 mM EDTA, pH 7.2), transferred to 1× λ PPase reaction buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM $MnCl_2$, pH 7.5), and divided into four aliquots of 50 μl. 1 μl λ PPase (NEB, P0753S) +/– PPase inhibitor (10 mM Na_3VO_4 or 50 mM EDTA) was added to the IP protein samples and incubated at 30°C for 15 minutes.

To assay for UNC-51 autophosphorylation, immunoprecipitated UNC-51, UNC-51(K39R) and UNC-51(ΔAIKAI) were exchanged into kinase buffer (50 mM Hepes, 10 mM $MgCl_2$, pH 7.5). The reaction was carried out in a 50 μl volume of kinase buffer plus 0.2 mM ATP, 10 mM sodium orthovanadate, protease inhibitors and 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol). Samples were incubated at 25°C for about 30 minutes and washed 4× with 0.1% Triton wash buffer. The bead pellets were resuspended with 40 μl 2× sample buffer.

To assay for UNC-14 phosphorylation, GST-UNC-14 fusion protein (encoded by pGEX-unc-14-HA) was expressed and purified as described above and washed 3× with kinase buffer. UNC-51-FLAG protein was immunoprecipitated from COS cell extract as described above and eluted with two washes of 120 μl of 1 mg/ml 3× FLAG peptide in elution buffer (20 mM Hepes, 100 mM KCl, 0.2 mM EDTA, 0.1% NP40, protease inhibitors, pH 7.7) at room temperature for 25 minutes each. Kinase reactions were carried out with 70 μl of UNC-51-FLAG elute plus 0.2 mM ATP, 10 mM $MgCl_2$ and 20 μCi [γ-^{32}P]ATP. Samples were incubated at 25°C for about 30 minutes and washed 4× with 0.1% Triton wash buffer.

Protein samples were denatured at 85-90°C for 10 minutes, and separated on 8% SDS-PAGE gel. The gel was dried onto Whatman paper and signals were processed with a phospho-imager (Molecular Dynamics, Storm 820). Incorporation of [γ-^{32}P]ATP was determined by measuring band intensity using the Image Quant 5.2 software (Molecular Dynamics).

unc-51 RNAi

unc-51 cDNA sequences corresponding to aa 1-410 were subcloned into the L4440 vector (containing two T7 transcription start sites, Fire lab 1999 vector kit). The resulting construct was L4440-U51RNAi1. dsRNA was prepared and injected using standard procedures (Fire Lab RNAi protocol, Version 1.0, www.ciwemb.edu/pages/firelab.html).

Results

VAB-8 and UNC-51 interact physically

We previously showed that the gene *vab-8* encodes two intracellular proteins that are required for most posteriorly directed cell and growth cone migrations in *C. elegans* (Wightman et al., 1996; Wolf et al., 1998). To better understand the role of *vab-8* in growth cone guidance, we conducted a yeast two-hybrid screen to identify VAB-8-interacting proteins. Using full-length VAB-8 protein as bait, we screened a library of mixed stage *C. elegans* cDNAs. One positive clone contained sequences from the gene *unc-51*, a promising candidate because *unc-51* mutants display axon defects and because VAB-8 is expressed in a subset of UNC-51-expressing neurons (Ogura et al., 1994; Wolf et al., 1998).

Using a yeast two-hybrid binding assay, we defined the C-terminal 106 amino acids of UNC-51 as sufficient to bind VAB-8 (Fig. 1A). Using a GST pulldown assay, we showed that the interaction between VAB-8 and UNC-51 was direct and defined two regions of VAB-8 that can bind UNC-51 (Fig. 1B,C). Ogura et al. (Ogura et al., 1997) had shown that the C-terminal 401 amino acids of UNC-51 bound UNC-14, and we found that the same 106 amino acid region of UNC-51 that bound VAB-8 also bound UNC-14 (Fig. 1D; data not shown).

vab-8 and *unc-51* mutant phenotypes

In addition to the physical interaction described above, the overlapping mutant phenotypes and expression patterns of *vab-8* and *unc-51* suggest that the two proteins may function together in vivo. *vab-8* mutations are more specific, disrupting only posteriorly directed axon outgrowth (Wightman et al., 1996; Wolf et al., 1998), while *unc-51* mutations disrupt the outgrowth of axons along both the anteroposterior and dorsoventral axis (Hedgecock et al., 1985; McIntire et al., 1992). Consistent with a more specific role in axon guidance,

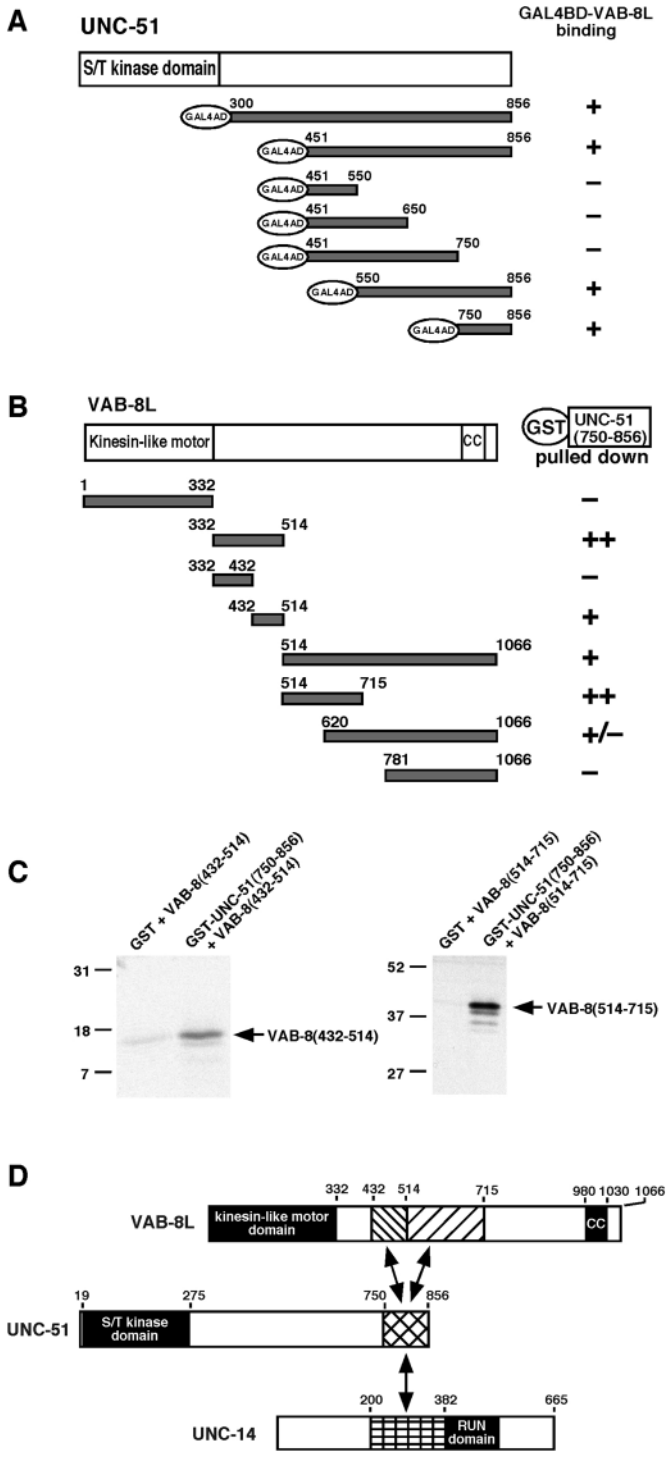

Fig. 1. VAB-8 and UNC-51 interactions. (A) Yeast two-hybrid interactions between full-length VAB-8 and various UNC-51 fragments. VAB-8L was fused to the GAL4 DNA binding domain (GAL4BD), and UNC-51 fragments were fused to the GAL4 activation domain (GAL4AD). Binding was determined by β-galactosidase activity using filter lift assays (Durfee et al., 1993). +, most colonies had turned blue overnight; –, no colony had turned blue overnight. (B) GST and GST-UNC-51(750-856) fusion proteins were expressed and purified from *E. coli,* and bound to glutathione-conjugated beads. Various protein domains of VAB-8 were transcribed and translated in vitro using reticulocyte lysates (see Materials and methods). The + and – signs represent the strength of binding of each VAB-8 fragment to UNC-51(750-856) as compared to GST alone. (C) Domains of VAB-8 sufficient to bind UNC-51(750-856). (D) Schematic representation of binding interactions between UNC-51 and both VAB-8 and UNC-14. The hatched boxes represent the domains that are sufficient for binding. VAB-8 contains two domains that are sufficient to bind to UNC-51.

vab-8 is expressed in a subset of the neurons that express *unc-51* (Ogura et al., 1994; Wolf et al., 1998) (data not shown). To study the axon phenotypes of the mutants further, we focused on the CAN axons, which can be visualized using transgenes that express GFP from the *ceh-23* promoter (Forrester and Garriga, 1997; Zallen et al., 1999). Animals bearing a *Pceh-23::gfp* transgene expressed GFP in the CANs and in several sensory neurons located in the head and tail (Fig. 2A,B). The CANs are a pair of neurons that migrate from their birthplace in the head to the middle of the embryo (Sulston et al., 1983). After reaching their destinations, each CAN extends an axon anteriorly to the head and then an axon posteriorly to the tail (J. Withee and G.G., unpublished observations).

In mutants lacking *vab-8* function, CAN cell migration and posteriorly directed axon guidance fail. CAN cell migration requires the short form of VAB-8, VAB-8S, while posteriorly directed axon guidance requires the long form, VAB-8L. To assess the role of *vab-8* in CAN axon guidance, we used the *vab-8(ev411)* mutation, which disrupts the function of VAB-8L, but not VAB-8S (Wolf et al., 1998). In *ev411* mutants, the CANs migrated and extended their anteriorly directed axons normally, but their posteriorly directed axons often terminated prematurely (Fig. 2C) (Wightman et al., 1996) or turned around and extended anteriorly (Fig. 2D). *unc-51* mutations resulted in more general defects in CAN axon outgrowth. We show here the phenotype of *e369* (Fig. 2E,F), the strongest *unc-51* allele by phenotypic and molecular criteria (Ogura et al., 1994) (data not shown). The mutation affected extensions of both anteriorly and posteriorly directed axons, but did not cause a misrouting defect. In addition, the *unc-51* mutation often resulted in two large varicosities that flanked the cell body (Fig. 2E,F). Unusually large varicosities have been described for other *unc-51* axons as well (Hedgecock et al., 1985; McIntire et al., 1992).

We analyzed *vab-8(ev411) unc-51(e369)* double mutants and observed that the CAN posterior axon defect was worse in the double than in either single mutant (data not shown). The *e369* mutation does not appear to eliminate *unc-51* activity as the phenotypes it caused could be enhanced by *unc-51* RNAi (data not shown). Thus, we could not use the increased severity of the *vab-8(ev411) unc-51(e369)* mutant phenotypes to determine whether VAB-8 and UNC-51 act in the same or separate pathways. We also examined the AVK axons, which extend posteriorly along the ventral nerve cord (White et al., 1986). In *vab-8* or *unc-51* mutants, AVK axons stopped short at various points along the ventral nerve cord (Wolf et al., 1998) (data not shown). In *vab-8 unc-51* double mutants, the AVK axon defect was more severe (data not shown).

vab-8 and *unc-51* act cell autonomously

If the interaction between VAB-8 and UNC-51 is functionally important for axon guidance, both proteins should be required in the same cell. To test whether both genes function cell

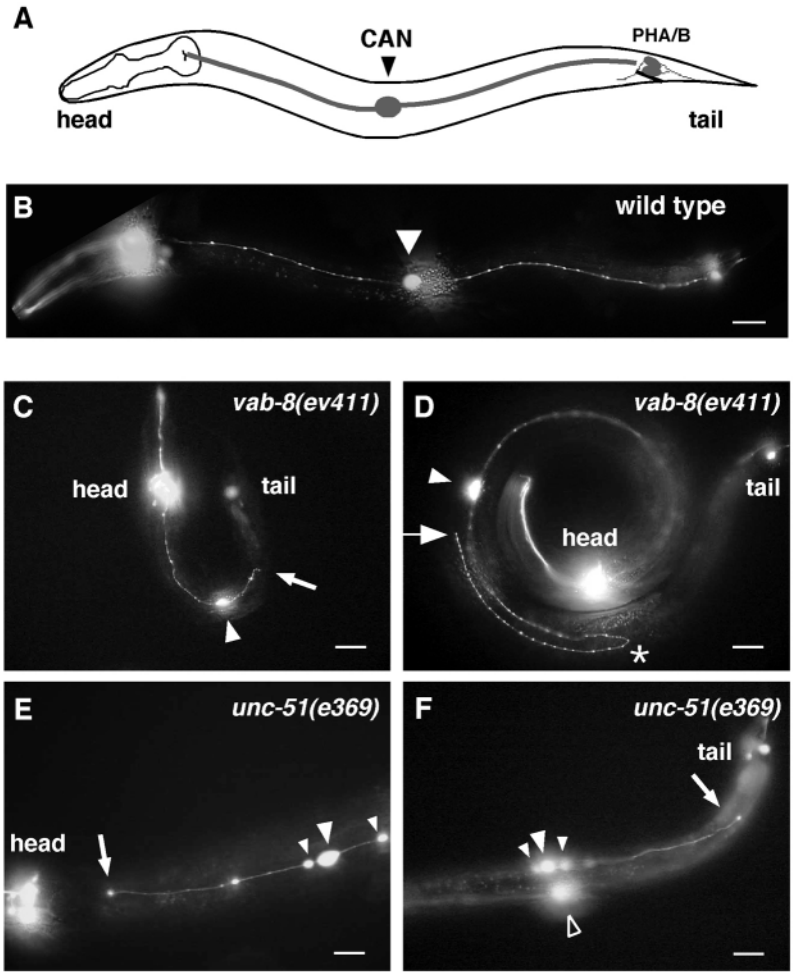

Fig. 2. CAN axon morphology in wild-type, *vab-8* and *unc-51* animals. (A) Schematic representation of a CAN cell body and its axons. The centrally positioned CAN cell body extends an axon anteriorly to the nerve ring and an axon posteriorly to the tail near the PHA/B sensory neurons. (B-F) Fluorescence photomicrographs of larvae carrying a *Pceh-23::gfp* transgene, which expresses GFP in the CANs, as well as sensory neurons in the head and the tail. The CAN cell bodies (large arrowheads) and their axons can be visualized using this transgene. An arrow indicates the position of an axon termination. (B) Wild-type third larval stage hermaphrodite. (C) *vab-8(ev411)* first larval stage hermaphrodite. (D) *vab-8(ev411)* fourth larval stage hermaphrodite. The asterisk indicates the position where the posterior axon had reversed and extended anteriorly. The penetrance of this misrouting defect is 29% for *ev411*. (E,F) The small arrowheads point to large varicosities that often flank the CAN cell bodies in *unc-51* mutants. (E) Anterior half of an *unc-51(e369)* third larval stage hermaphrodite. (F) Posterior half of a different *unc-51(e369)* larva. The open arrowhead points to an out-of-focus CAN cell body. Scale bars: 20 μm.

autonomously, we expressed the long form of *vab-8* in the CAN from the *ceh-23* promoter and found that this transgene partially rescued both the early termination and misrouting defects of *vab-8(ev411)* posterior CAN axons (Fig. 3; data not shown). Expression of an *unc-51* cDNA from the *ceh-23* promoter rescued both the anterior and posterior CAN axon extension defects, as well as the axon varicosity defect of *unc-51* mutants (Fig. 3; data not shown). Even though these transgenes also expressed *vab-8* or *unc-51* in several sensory neurons, it seems unlikely that expression from these cells was responsible for rescue of the CAN defects. First, these sensory neurons project their axons along trajectories that are distinct from those of the CAN axons. Second, neither VAB-8 nor UNC-51 is a secreted molecule that can act over a long distance. Thus, we propose that both VAB-8 and UNC-51 function in the CAN cells to promote the directed outgrowth of their axons.

Expression of VAB-8 and UNC-51 binding domains disrupts CAN axon outgrowth

We have shown that VAB-8 and UNC-51 are required in the CAN for proper axon outgrowth and that in vitro the two proteins can physically interact. To test the hypothesis that the interaction between VAB-8 and UNC-51 is functionally important, we attempted to disrupt this interaction in vivo by expressing the interacting domains of these two proteins in the CANs. We reasoned that the VAB-8 peptide might compete with endogenous wild-type VAB-8 for UNC-51 binding and thus interfere with axon outgrowth. As predicted, expressing the UNC-51-binding domain of VAB-8 (array *gmEx266*) in the CANs produced a posterior axon defect (Fig. 4A,B).

We also conducted the reciprocal experiment by expressing an UNC-51 fragment containing the VAB-8-binding domain (array *gmEx264*), which also resulted in a posterior axon defect (Fig. 4B). Expression of this peptide, however, also resulted in an occasional anterior axon truncation defect (data not shown). We initially generated and analyzed multiple array lines for each peptide construct, and because they behaved similarly, we have concentrated on one array for our analysis. To rule out the possibility that the defect we observed was a nonspecific effect of high levels of the *ceh-23* promoter, we expressed various cDNAs from this promoter and saw no CAN axon defects (data not shown).

The posterior axon defects were partially suppressed by the simultaneous expression of both protein peptides (*gmEx266* and *gmEx264*; Fig. 4B), which presumably bind to each other to allow more of the endogenous wild-type VAB-8 and UNC-51 proteins to interact. To rule out the possibility that this suppression was due to increased levels of the *ceh-23* promoter in animals bearing both transgenes, we also constructed animals that had *gmEx266* and an additional array that contained an equivalent amount of the *ceh-23* promoter as *gmEx264*. Addition of this array had no effect on the CAN defects induced by *gmEx266*, ruling out the possibility that suppression was caused by the depletion of *ceh-23* transcriptional activators (data not shown). Taken together, the peptide expression experiments suggest that the VAB-8 and UNC-51 interaction in the CAN neuron is required for the directed outgrowth of its posterior axon.

The VAB-8 peptide also disrupts the interaction between UNC-51 and UNC-14

If the peptides only disrupted the interaction between VAB-8

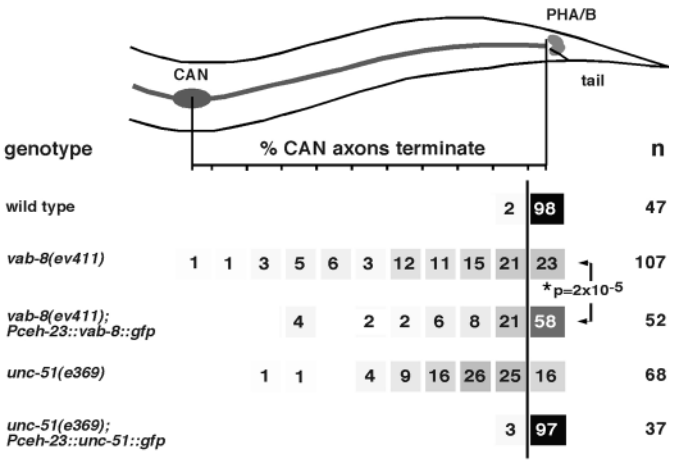

Fig. 3. *vab-8* and *unc-51* act autonomously in the CAN cell. *vab-8* or *unc-51* full-length cDNAs was expressed from the *ceh-23* promoter. At the top is a schematic representation of the posterior half of *C. elegans*, showing a CAN cell body and its posterior axon. Also shown are two sensory neurons (PHA/B) that express the *Pceh-23::gfp* transgene and mark the position where the CAN axon terminates. We scored the extent to which the posterior axons extended, with the position of the CAN cell body representing 0% extension and the position of the PHA/B sensory neurons representing 100% extension (see Materials and methods). The numbers in the boxes represent the percentages of axons that terminated or turned in that interval. Axons that completed 95-100% of the distance from the CAN cell body to the sensory neurons are considered wild type. n is the number of axons scored. To simplify the statistical analysis, only percentages of the wild-type axons (to the right of the vertical line) were compared. *The two-tailed Z test was used to compare the differences in the distributions of the two populations of axons in the wild-type position.

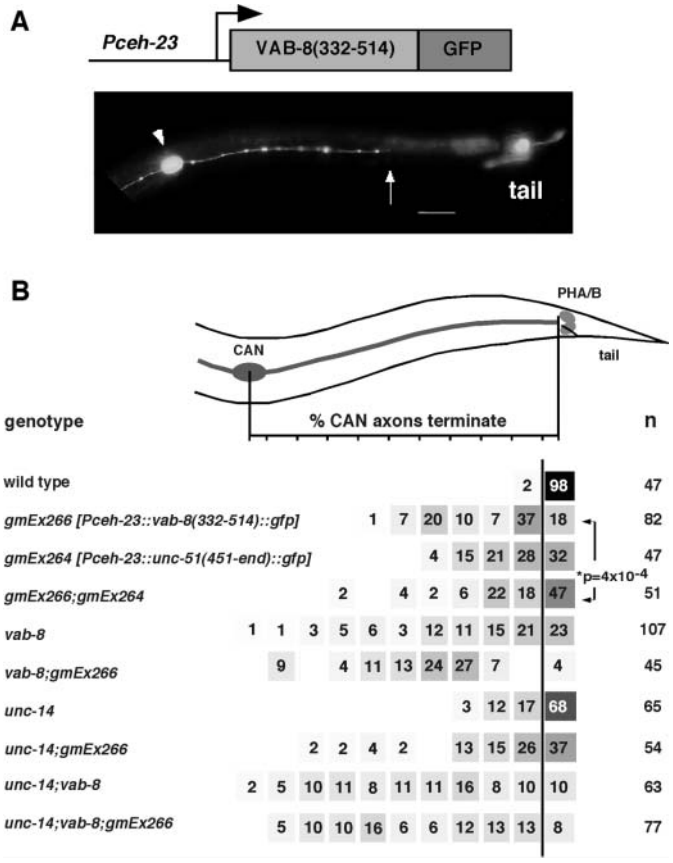

Fig. 4. Expression of the binding domains of VAB-8 and UNC-51 in the CANs disrupted posteriorly directed axon outgrowth. (A) At the top is a schematic representation of the transgene that expresses the VAB-8 peptide in the CANs. A cDNA containing this UNC-51-binding domain of VAB-8 was fused in frame to a GFP cDNA and driven from the *ceh-23* promoter. The fluorescence photomicrograph shows a wild-type larva that carries this transgene. The arrowhead indicates the position of the CAN cell body, and the arrow indicates the end of its truncated posterior axon. The scale bar represents 20 μm. (B) The distribution of axon termination positions in wild type, mutants and animals that expressed VAB-8 and/or UNC-51 peptides in the CANs. The *vab-8* allele used was *ev411*; the *unc-14* allele used was *e866*. See Fig. 3 for quantification of axon phenotypes and statistical analysis.

and UNC-51, we reasoned that their expression should not enhance a mutant completely lacking *vab-8* or *unc-51* function. Since no *unc-51* null allele exists (see above), we expressed the VAB-8 peptide in *vab-8(ev411)*, a mutant that lacks VAB-8L function (Wightman et al., 1996; Wolf et al., 1998). We found that expression of the VAB-8 peptide could further enhance the posterior CAN axon defect of the mutant (Fig. 4B). One possible explanation for this enhancement is that this peptide interfered with the binding of UNC-51 to molecules other than VAB-8. One candidate molecule is UNC-14, which was shown by Ogura et al. to bind the C-terminal half of UNC-51, (Ogura et al., 1997). We refined the UNC-14-binding region to the C-terminal 106 amino acids of UNC-51 (data not shown), the same region that binds to VAB-8. We analyzed two alleles of *unc-14, e866* (Fig. 4B) and *e1119* (data not shown). Both alleles are likely to be nulls as they are nonsense mutations near the beginning of the open reading frame. Both *unc-14* mutations caused a CAN posterior but not an anterior truncation defect.

To test the possibility that the VAB-8 peptide disrupted interactions between UNC-51 and both VAB-8 and UNC-14, we asked whether expression of the VAB-8 peptide would enhance the posterior CAN axon defect of an *unc-14(e866); vab-8(ev411)* double mutant. *unc-14(e866)* is a nonsense mutation at amino acid 106, and thus eliminates the UNC-51-binding domain. As expected, the posterior CAN axon defect was more severe in the *unc-14(e866); vab-8(ev411)* double mutant than in either single mutant alone, demonstrating that VAB-8 and UNC-14 function in distinct processes required for axon outgrowth (Fig. 4B). Expression of the VAB-8 peptide, however, did not enhance the posterior axon defect of the double mutant (Fig. 4B). This result is consistent with the interpretation that the VAB-8 peptide disrupted the interaction of UNC-51 with both VAB-8 and UNC-14, and in the absence of both of these proteins, expression of the VAB-8 peptide had no effect. The lack of enhancement did not result from an inability to generate a more severe phenotype, as we have observed more severe axon defects in other mutant backgrounds (data not shown). It is noteworthy that the VAB-8 peptide effect was weakly suppressed in the *unc-14* mutant background (Fig. 4B). This result could be explained if the interaction of UNC-51 with VAB-8 plays a larger role in CAN

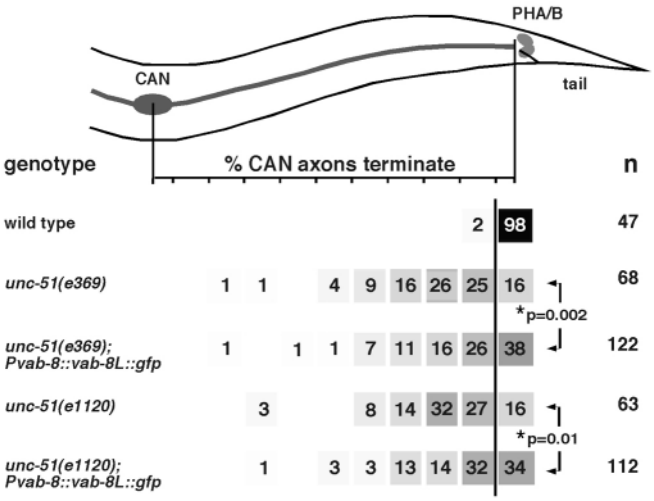

Fig. 5. Overexpression of *vab-8L* partially suppressed the CAN posterior axon outgrowth defect of *unc-51* mutants. A full-length *vab-8* cDNA was expressed from the *vab-8* promoter. See Fig. 3 for quantification of axon phenotypes and statistical analysis.

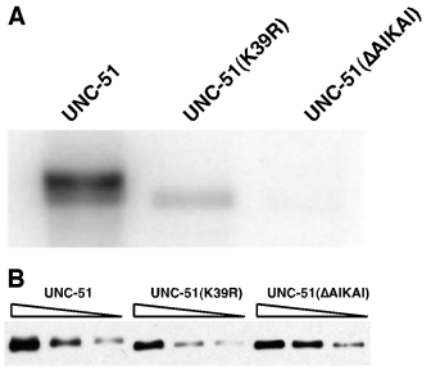

Fig. 6. UNC-51 autophosphorylation. (A) Autoradiograph of samples from in vitro kinase assays. FLAG-tagged wild-type and mutant UNC-51 proteins were immunoprecipitated from COS cell extracts and incubated with [γ-^{32}P]ATP. UNC-51(K39R) had 9% and UNC-51(ΔAIKAI) had 0.6% of the wild-type UNC-51 autophosphorylation activity. See Materials and methods for quantification of kinase activity. (B) Loading control. The western blot was probed with anti-FLAG antibodies to detect various forms of UNC-51 proteins. Similar amounts of immunoprecipitated UNC-51 proteins were used in the kinase assay.

axon outgrowth, so that in the absence of UNC-14, more UNC-51 protein is available to interact with VAB-8.

Overexpression of *vab-8* suppresses the posterior CAN axon defect of *unc-51* mutants

Our results indicate that the physical interaction between VAB-8 and UNC-51 is important for the functions of these proteins in axon outgrowth. One of these proteins could activate the other, or alternatively, one could inhibit the function of the other. To distinguish between these possibilities, we overexpressed *vab-8* in *unc-51* mutants and found that *vab-8* overexpression suppressed the posterior CAN axon defect of *unc-51(e369)* and *unc-51(e1120)* mutants (Fig. 5). The *vab-8* mini gene used in these experiments was previously shown to result in VAB-8 overexpression when present in extrachromosomal arrays (Wolf et al., 1998). Our result suggests that *vab-8* and *unc-51* act in a positive regulatory pathway. Ordinarily, we would be unable to order the genes based on this genetic result because neither of the *unc-51* alleles appears to be null. None of the characterized *unc-51* mutations is a large deletion, early nonsense or frameshift mutation, or is in the kinase domain (Ogura et al., 1994). Furthermore, *unc-51* RNAi was able to enhance the CAN axon defect of *unc-51(e369*) (data not shown), the most severe *unc-51* mutant phenotypically and molecularly (Ogura et al., 1994) (data not shown). *e369*, however, contains an amber nonsense mutation predicted to eliminate the VAB-8-binding site in UNC-51. The ability of *vab-8* overexpression to suppress this *unc-51* mutation demonstrates that increased VAB-8 levels can bypass the requirement for the VAB-8 and UNC-51 interaction, and suggests that *vab-8* functions downstream of *unc-51*. We could not determine the effect of *unc-51* overexpression on *vab-8*, because the transgenic array containing *unc-51* full-length cDNA did not express UNC-51 stably.

UNC-14 and VAB-8 are substrates for UNC-51 kinase activity

Tomoda et al. (Tomoda et al., 1999) have reported autophosphorylation of the mouse UNC-51 ortholog. Incubation of recombinant *C. elegans* UNC-51 with [γ-^{32}P]ATP in vitro also resulted in autophosphorylation (Fig. 6A). An UNC-51(K39R) mutant protein, which alters a conserved lysine usually essential for kinase activity (Hanks et al., 1988), retained approximately 9% of the kinase activity of wild-type UNC-51, while an UNC-51(ΔAIKAI) mutant protein, which lacks the same lysine and four flanking amino acids, possessed no detectable kinase activity (Fig. 6).

To explore the possibility that UNC-14 and VAB-8 could be UNC-51 substrates, we expressed either UNC-14 or VAB-8 with UNC-51 in COS cells. When we expressed UNC-14 in the presence of UNC-51, we observed a shift of UNC-14 in western blots to a series of higher molecular mass bands that could be reversed by phosphatase treatment (Fig. 7A,B). This reversal was sensitive to phosphatase inhibitors (Fig. 7B). Expression of UNC-14 with the UNC-51(K39R) mutant resulted in a loss of most of the phosphorylated isoforms, although we observed a small shift in UNC-14 molecular mass (Fig. 7A). As this UNC-51 protein retains partial activity, we propose that the K39R mutant protein can phosphorylate UNC-14 inefficiently. Expression of UNC-14 with the UNC-51(ΔAIKAI) mutant resulted in the loss of all phosphorylated isoforms (Fig. 7A). Since this mutant protein lacks activity in vitro, this result is consistent with UNC-14 phosphorylation requiring UNC-51 kinase activity. To demonstrate that UNC-14 is a direct target of UNC-51, we performed an in vitro kinase assay on GST-tagged UNC-14 expressed and purified from bacteria. UNC-51 protein expressed and purified from COS cells was able to phosphorylate GST-UNC-14 in vitro (Fig. 7C).

Co-expression of VAB-8 and UNC-51 in COS cells also resulted in a shift of VAB-8 to a series of higher molecular mass bands in western blots that could be reversed by phosphatase treatment (Fig. 7D,E). The UNC-51(K39R) mutant protein resulted in a level of VAB-8 phosphorylation that was similar to that seen with wild-type UNC-51 (Fig. 7D). One possible explanation for this observation is that VAB-8 is

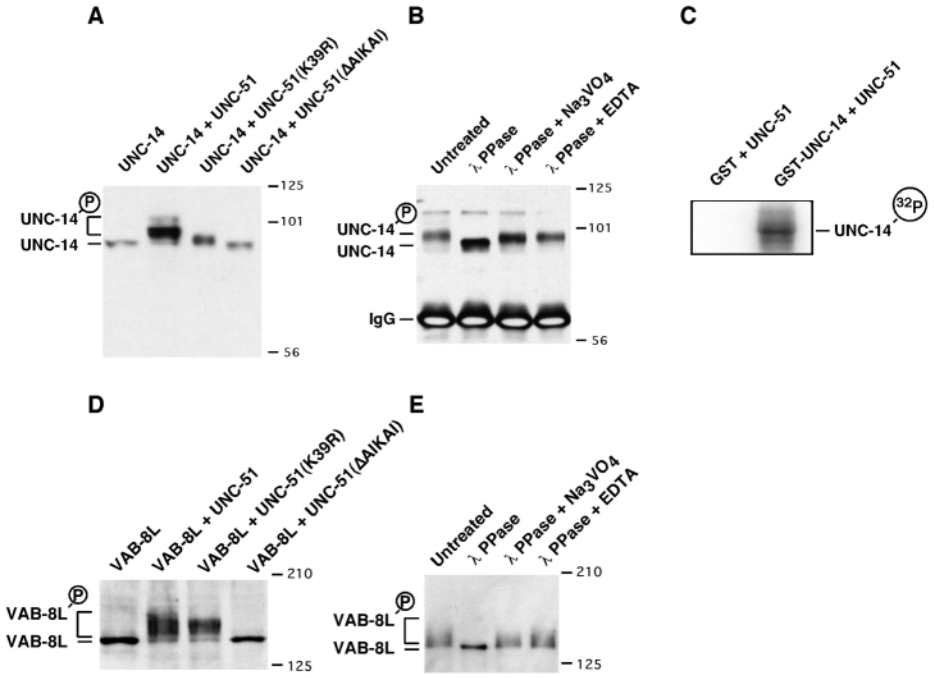

Fig. 7. UNC-51-dependent phosphorylation of UNC-14 and VAB-8. (A,B) Western blots were probed with anti-HA antibodies to detect UNC-14. (A) COS cell extracts expressing UNC-14 alone, or with wild-type or mutant UNC-51 proteins. UNC-14 was phosphorylated when coexpressed with wild-type UNC-51, partially phosphorylated when coexpressed with UNC-51(K39R) and not phosphorylated when coexpressed with UNC-51(ΔAIKAI). (B) All lanes contained UNC-14 that was immunoprecipitated from COS cells expressing both wild-type UNC-51 and UNC-14. The sample from the second lane was treated with λ phosphatase (λ PPase) to yield a lower molecular mass band that ran at the same size as the product from cells expressing only UNC-14. Adding the phosphatase inhibitors Na_3VO_4 or EDTA inhibited the ability of λ PPase to yield the lower molecular mass UNC-14 band. (C) GST and GST-UNC-14 proteins were expressed and purified from *E. coli*, and incubated with [γ-^{32}P]ATP and wild-type UNC-51 purified from COS cells. GST-UNC-14 was labeled, whereas no labeling was seen with the GST control. (D,E) Western blots were probed with anti-Myc antibodies to detect VAB-8. (D) COS cell extracts expressing VAB-8 alone, or with wild-type or mutant UNC-51 proteins. VAB-8 was phosphorylated when coexpressed with wild-type UNC-51 or with UNC-51(K39R), but not phosphorylated when coexpressed with UNC-51(ΔAIKAI). (E) All lanes contained VAB-8 that was immunoprecipitated from COS cells that expressed both wild-type UNC-51 and VAB-8. λ PPase treatment of the sample in the second lane converted multiple bands into one lower molecular mass band that ran at the same size as the product from cells expressing only VAB-8. Adding the phosphatase inhibitors Na_3VO_4 or EDTA inhibited the ability of λ PPase to yield the lower molecular mass VAB-8 band.

a better UNC-51 substrate than UNC-14, and the partially active kinase can result in efficient phosphorylation. Alternatively, VAB-8 could be a poor substrate for phosphatases in COS cells that dephosphorylate UNC-14 efficiently. VAB-8 phosphorylation was completely eliminated when coexpressed with UNC-51(ΔAIKAI) (Fig. 7D). Since this mutant protein lacks activity in vitro, our results are consistent with VAB-8 being a target of UNC-51 kinase activity. Our attempts to demonstrate that VAB-8 is a direct substrate for purified UNC-51 were hindered by our inability to purify intact VAB-8 protein.

Discussion

We report here that VAB-8 and UNC-51 can physically interact. By focusing on the outgrowth of the CAN axons, we made several observations indicating that the interactions between the two proteins are functionally important for axon outgrowth in *C. elegans*. First, both proteins function in the CAN neurons to promote normal axon outgrowth. Second, expression in the CANs of peptides predicted to disrupt the VAB-8 and UNC-51 interaction interfered with axon outgrowth. Third, overexpression of VAB-8 partially suppressed the posterior axon defect of *unc-51* mutants. Finally, UNC-51 could phosphorylate VAB-8 in COS cells. Taken together, our results suggest that UNC-51 regulates the activity of VAB-8 in posteriorly directed axon outgrowth.

During the course of these experiments, we discovered that a peptide predicted to disrupt interactions between UNC-51 and VAB-8 also disrupted the interaction between UNC-51 and UNC-14. We showed that UNC-51 could phosphorylate UNC-14, suggesting that UNC-51 regulates the activity of UNC-14. To our knowledge, VAB-8 and UNC-14 are the first proteins identified as targets of the UNC-51 kinase family. Based on our genetic and molecular results, we propose that VAB-8 and UNC-14 mediate the effects of UNC-51 to regulate posteriorly directed axon outgrowth.

The UNC-51 protein family appears to function in membrane trafficking

UNC-51 is a conserved kinase with homologs found in organisms as diverse as yeast and humans. The yeast homolog of UNC-51, Apg1p, is required for the cytoplasm-to-vacuole (Cvt) targeting pathway, which is involved in the transport of the vacuolar hydrolase aminopeptidase I, and for the induction of autophagy, a process that involves the delivery of organelles and cytoplasm to the lysosome (for reviews, see Huang and Klionsky, 2002; Noda et al., 2002; Reggiori and Klionsky, 2002). Both the Cvt pathway and autophagy require the de novo synthesis of cup-shaped membrane cisternae, which elongate and fuse to form double-membraned Cvt vesicles and autophagosomes. Apg1p, complexed with different binding partners, is proposed to regulate the formation of these membrane structures (Noda et al., 2002).

While VAB-8 and UNC-14 are the first putative phosphorylation targets of UNC-51 identified, several other proteins have been shown to physically interact with UNC-51 homologs. Humans have two UNC-51-like kinases, ULK1 and ULK2 (Kuroyanagi et al., 1998; Yan et al., 1998; Yan et al., 1999). In a yeast two-hybrid screen for proteins that

interact with human ULK1, Okazaki et al. (Okazaki et al., 2000) identified both the $GABA_A$ receptor associated protein (GABARAP) and the Golgi-associated ATPase enhancer of 16 kDa (GATE-16). GABARAP was originally identified as a protein that could physically interact with the γ2 subunit of the $GABA_A$ receptor and was found to co-localize with this receptor in cultured cortical neurons (Wang et al., 1999). GABARAP shares similarity with microtubule-associated proteins and can bind microtubules (MTs), raising the possibility that it provides a link between MTs and the $GABA_A$ receptor (Wang et al., 1999). GATE-16 is an essential component for intra-Golgi transport and regulates SNARE function (Muller et al., 2002; Sagiv et al., 2000). GABARAP and GATE-16 are related to one another, and to the yeast autophagic factor Aut7p. Taken together, these observations suggest that UNC-51 homologs and their interacting proteins could function in membrane dynamics and vesicle trafficking.

As with *C. elegans* UNC-51, its mammalian homologs also regulate neuronal development. Mouse Ulk1 (a.k.a. Unc51.1) has been implicated in neurite outgrowth of cerebellar granule cells, suggesting that the function of UNC-51 homologs in axon outgrowth is conserved (Tomoda et al., 1999). In a yeast two-hybrid screen, Unc51.1 was found to interact with SynGAP, a Ras GAP, and syntenin, a PDZ domain-containing protein (Tomoda et al., 2004). SynGAP was also found to be a GAP for Rab5, and syntenin binds Rab5. Unc51.1 can downregulate SynGAP, leading to Rab5 activation, suggesting that a complex of Unc-51.1, SynGAP and syntenin controls axon outgrowth through its regulation of Rab5 activity (Tomoda et al., 2004). Since axon outgrowth requires membrane synthesis and vesicle trafficking to deliver cellular components necessary for the formation and steering of the growth cone, UNC-51 and its homologs could regulate these processes.

UNC-51 and UNC-14 also function in membrane trafficking

The requirement of Apg1p in autophagy and the interactions between UNC-51 homologs and proteins involved in receptor and membrane trafficking, suggest a conserved role for UNC-51-like kinases. The Unc-51 phenotypes described in *C. elegans* support this hypothesis. *unc-51* mutant animals have axons that form unusually large varicosities that accumulate internal membrane structures, including abnormal vesicles and cisternae-like structures (McIntire et al., 1992). The same study found similar but less severe defects in *unc-14* mutants. Based on these observations, McIntire et al. (McIntire et al., 1992) proposed that UNC-51 and UNC-14 functioned together in membrane trafficking. The functional link between UNC-51 and UNC-14 was strengthened by the ability of the two proteins to physically interact (Ogura et al., 1997). Our results suggest that UNC-51 can regulate the activity of UNC-14.

UNC-14 contains a RUN domain (Callebaut et al., 2001). While the function of this domain is unknown, several RUN domain-containing proteins are linked to the functions of Rab and Rap GTPases, and appear to function in vesicular trafficking (Callebaut et al., 2001; Mari et al., 2001; Yang et al., 2002), supporting the hypothesis that UNC-14 regulates membrane trafficking.

The role of VAB-8 and UNC-51 in axon outgrowth

Our results suggest that VAB-8 mediates the function of UNC-51 in axon guidance. The role of VAB-8 in posteriorly directed migrations, however, remains enigmatic. Although VAB-8 may act within an UNC-51 pathway to mediate vesicle transport, *vab-8* mutants lack the prominent axon varicosities displayed by *unc-51* and *unc-14* mutants. Alternatively, UNC-51 could act with VAB-8 in a distinct process required for directed axon outgrowth. VAB-8 has been placed into a subfamily of kinesin-like molecules that includes *Drosophila* Costal2 (Miki et al., 2001). Although this molecule appears to lack kinesin motor activity, it retains an ability to bind to MTs and serves as a cytoplasmic tether for the ci transcription factor in Hedgehog signaling (Sisson et al., 1997). Perhaps as proposed for GABARAP, VAB-8 could provide a link between the cytoskeleton and guidance receptors. Given the specificity of VAB-8 in regulating posteriorly directed migrations, one interesting speculation is that VAB-8 regulates the activity of receptors involved in posteriorly directed guidance.

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