Abl Tyrosine Kinase and Its Substrate Ena/VASP Have Functional Interactions with Kinesin-1[□]

MaryAnn Martin,^{*†} Shawn M. Ahern-Djamali,^{†‡} F. Michael Hoffmann,[‡] and William M. Saxton^{*}

^{*}Department of Biology, Indiana University, Bloomington, IN 47405; and [†]McArdle Laboratory, University of Wisconsin, Madison, WI 53706

Submitted February 10, 2005; Revised June 13, 2005; Accepted June 15, 2005
Monitoring Editor: John Pringle

Relatively little is known about how microtubule motors are controlled or about how the functions of different cytoskeletal systems are integrated. A yeast two-hybrid screen for proteins that bind to *Drosophila* Enabled (Ena), an actin polymerization factor that is negatively regulated by Abl tyrosine kinase, identified kinesin heavy chain (Khc), a member of the kinesin-1 subfamily of microtubule motors. Coimmunoprecipitation from *Drosophila* cytosol confirmed a physical interaction between Khc and Ena. Kinesin-1 motors can carry organelles and other macromolecular cargoes from neuronal cell bodies toward terminals in fast-axonal-transport. Ena distribution in larval axons was not affected by mutations in the *Khc* gene, suggesting that Ena is not itself a fast transport cargo of *Drosophila* kinesin-1. Genetic interaction tests showed that in a background sensitized by reduced *Khc* gene dosage, a reduction in *Abl* gene dosage caused distal paralysis and axonal swellings. A concomitant reduction in *ena* dosage rescued those defects. These results suggest that Ena/VASP, when not inhibited by the Abl pathway, can bind Khc and reduce its transport activity in axons.

INTRODUCTION

The activities of the F-actin and microtubule cytoskeletons are linked in cellular processes such as secretion, cytokinesis, and axon outgrowth, but relatively little is known about mechanisms that coordinate the activities of the two filament systems. The nonreceptor tyrosine kinase Abl, aberrant forms of which are implicated in human leukemia, influences axon outgrowth and other F-actin-dependent processes (Woodring *et al.*, 2003; Hernandez *et al.*, 2004). Recent work suggests that Abl also influences microtubule polymerization in the axon growth cone via interactions with Orbit and that a mouse Abl-related protein, Arg, can cross-link F-actin with microtubules in the cell periphery (Lee *et al.*, 2004; Miller *et al.*, 2004). In part, the influence of Abl on F-actin-dependent processes is mediated by its regulatory interaction with Ena/VASP proteins, which can modulate actin filament length, branching pattern, and bundle formation (reviewed by Krause *et al.*, 2003; Kwiatkowski *et al.*, 2003). Ena/VASP proteins have three conserved regions. The N-terminal EVH1 domain (133 amino acids, Gertler *et al.*, 1996) can bind the focal-adhesion proteins vinculin and zyxin, as well as the growth-cone guidance receptor Robo/Sax3. The central proline-rich region can bind profilin, which facilitates the addition of G-actin monomers to F-actin plus-

ends. It can also bind Abl and other SH3-domain proteins. The C-terminal EVH2 domain (226 amino acids, Gertler *et al.*, 1996) has both G- and F-actin binding sites and has been shown to mediate Ena/VASP multimerization (reviewed by Krause *et al.*, 2003; Kwiatkowski *et al.*, 2003).

In *Drosophila* development, Ena and Abl are known to have an antagonistic relationship. Zygotic mutations in the *ena* or *Abl* gene cause axon growth-cone guidance defects and lethality, but normal axon guidance and viability can be restored by combining *ena* and *Abl* mutations (reviewed by Krause *et al.*, 2003). Mutation of the *Abl* gene in the female germline eliminates maternal Abl protein from embryos, resulting in aberrant epithelial cell shape and defective dorsal closure. In embryonic epithelial cells that lack Abl, Ena mislocalization to the apical region causes overgrowth of F-actin based microvilli (Grevengoed *et al.*, 2001, 2003). Overall, it is evident that Ena protein helps form long, unbranched actin filaments and that Abl negatively regulates Ena activity and/or localization. Whether Abl controls Ena by simply binding it, by tyrosine phosphorylation, or by some other means remains unclear (reviewed by Krause *et al.*, 2003).

Kinesin-1 proteins are classic, plus-end-directed microtubule motors that function in mRNA localization, organelle movement, and axonal transport (reviewed by Vale, 2003). Kinesin-1s are not known to have direct roles in cell shape changes, axon guidance, or cell migration. Through protein-binding and genetic tests, we have identified physical and functional interactions between *Drosophila* kinesin-1, Abl, and Ena. Our results suggest that in addition to its influence on actin cytoskeleton organization, Abl signaling can influence microtubule-based anterograde fast transport in neurons by regulation of Ena, which binds the stalk-tail region of Khc. That region of Khc is thought to be important for kinesin-1 autoregulation and cargo interaction.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-02-0116>) on June 22, 2005.tk;2

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

[†] These authors contributed equally to this work.

Address correspondence to: William Saxton (bsaxton@bio.indiana.edu).

MATERIALS AND METHODS

Fly Stocks and Behavior Tests

Fly stocks were maintained as described previously (Hurd and Saxton, 1996). Chromosomal-deletion strains and all other fly strains, unless noted otherwise, were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN). Ena protein was overexpressed in flies by generating strains carrying a *GAL4-UAS:ena* transgene, *P[UAS-ena, w+/1A1]* (Comer *et al.*, 1998), and one of the following drivers: 1) *Act5C-GAL4* (*P[w⁺mc = Ay-GAL4]25*), 2) *HSP70-GAL4* (*P[w⁺mc = GAL4-Hsp70.PB]2*), or 3) *elav-GAL4* (*P[w⁺mW.hs = GawB]elav^{C155}*).

To gain an unbiased view of the severity of larval posterior paralysis (tail flipping) as a function of genotype, 25–50 randomly selected wandering third-instar larvae from each test class were studied as described previously (Martin *et al.*, 1999). Each cross was done and scored at least twice, and tests of *Abl*, *ena*, and *Khc* genetic interactions were done double-blind with negative and positive controls included. Wandering third-instar larvae were scored as tail flippers if at least two posterior segments curved up away from the substrate during the crawling cycle.

Yeast Two-hybrid and S2 Expression Constructs

A “bait” cDNA encoding the C-terminal 243 amino acids of Ena fused to the GAL4 DNA-binding domain was used to screen *Drosophila* larval cDNAs (“prey”) fused to sequences encoding the GAL4 activation domain, as described previously (Ahern-Djamali *et al.*, 1999). Blue colonies were isolated and retested, and the prey constructs in positive clones were subjected to sequence analysis. Full-length Ena, truncated Ena, and Khc transfection constructs were made by ligating cDNAs (Drosophila Genomics Resource Center, <http://dgrc.cgb.indiana.edu/>) into the pPacPL expression vector (Ahern-Djamali *et al.*, 1998). Mutant *ena* cDNAs included one that truncated the C-terminal 52 codons (Ahern-Djamali *et al.*, 1998), and another cut at a unique *NcoI* site that truncated the C-terminal 243 codons (this publication).

Immunoprecipitation

Drosophila S2 cells (1×10^7), either nontransfected or cotransfected (Gertler *et al.*, 1995) with cDNAs that expressed Ena and Khc, were lysed for 30 min on ice in 1 ml of extraction buffer (Saxton *et al.*, 1991). Adult fly heads were detached by vigorously shaking flies after freezing at -80°C or in liquid nitrogen. The heads and bodies were sorted at -20°C by passage through a no. 20 sieve, which retained bodies, and a no. 40 sieve, which retained heads. Frozen heads were disrupted with a cold ceramic mortar and pestle and then suspended in 1 ml of 4°C extraction buffer for every 0.12 g of starting material. Lysate or homogenate was clarified by centrifugation at $15,000 \times g$ for 20 min and then at $30,000 \times g$ for 30 min. The resulting S2 or head cytosol was immunoprecipitated, as described previously (Gertler *et al.*, 1995). Briefly, 1 μg of anti-GFP (8367–1, Clontech, Mountain View, CA), 1 μg of anti-Ena-carboxy-terminal (Gertler *et al.*, 1989), 1 μg of anti-*Drosophila*-Khc (Cytoskeleton, Denver, CO), or 15 μl of Flyk2 anti-Khc (Saxton *et al.*, 1991) was added to 300- μl fractions of cytosol (~400 heads) and rocked for 1 h at 4°C . Protein A- or G-coated beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added, and the suspensions were rocked overnight at 4°C and then washed three times with 500 μl of extraction buffer and boiled in SDS-PAGE sample buffer. SDS-PAGE (7.5% gels), blotting, and staining were done as described previously (Saxton *et al.*, 1988; Gertler *et al.*, 1995), except that an ECL system was used for detection (Amersham, Piscataway, NJ). Primary antibodies used to detect Ena and Khc included rabbit anti-Khc R1.5 (Saxton *et al.*, 1988) and rabbit anti-Ena-amino-terminal (Gertler *et al.*, 1989), as well as the antibodies described above. Prestained molecular weight standards were included in the SDS-PAGE, and their positions were marked on blots before blocking and antibody incubations.

Immunolocalization and Microscopy

Larval dissection, fixation, immunostaining, and confocal fluorescence microscopy were done as described previously (Hurd and Saxton, 1996) except that before anti-Ena staining, formaldehyde-fixed larvae were postfixed in -20°C methanol for 10 min and then washed in phosphate-buffered saline with 0.01% Tween 20. Dissection, fixation, and staining of mutant and control larvae were done in the same dish, and then micrographs were generated with identical optical and electronic settings using a Bio-Rad MRC600 confocal microscope (Richmond, CA; Hurd and Saxton, 1996). The antibodies and dilutions used were rabbit anti-*Drosophila*-synaptotagmin at 1:500 (Littleton *et al.*, 1993), mouse anti-*Drosophila*-cysteine-string-protein at 1:250 (Zinsmaier *et al.*, 1994), and rabbit anti-Ena-amino-terminal or rabbit anti-Ena-carboxy-terminal at 1:100 (Gertler *et al.*, 1989). The secondary antibodies (at 1:500) were goat anti-rabbit-IgG:FITC (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit-IgG:ALEXA-488, and goat anti-mouse-IgG:ALEXA-594 (both from Molecular Probes, Eugene, OR). Images were manipulated using NIH Image and Adobe Photoshop 7.0 (San Jose, CA).

Figure 1. Ena and Khc yeast two-hybrid interaction. (A) A cartoon of *Drosophila* Ena (684 amino acids). EVH1 and EVH2 represent the Ena-VASP homology domains, and Proline represents the proline-rich region. The 243-amino-acid C-terminal region used to screen for Ena-interacting proteins in a yeast two-hybrid assay is indicated with a black line. The region of Ena necessary for immunoprecipitation of Khc (see Figure 2) is bounded by arrows. (B) A cartoon of *Drosophila* Khc (975 amino acids) that notes structural features and the prey fragment (black line) that interacted with the Ena-C-terminal bait.

RESULTS

A Physical Interaction Between Khc and Ena Proteins

To identify binding partners and potential functions for Ena, its 243 C-terminal amino acids were used as bait in a yeast two-hybrid screen of a *Drosophila* larval cDNA prey library (Figure 1A). Positive two-hybrid interactions were detected in yeast colonies carrying cDNAs that encoded profilin and Ena, as reported previously (Ahern-Djamali *et al.*, 1998, 1999), an RNA binding protein (lark), a putative laminin receptor/ribosomal S2 protein (stubarista), and the C-terminal 261 amino acids of Khc (Figure 1B). That portion of Khc includes the alpha helical “coil-2” and globular “tail” regions that bind kinesin light chain (Klc), that mediate cargo interaction, and that can inhibit ATPase activity of the N-terminal motor domain (reviewed by Verhey and Rapoport, 2001). Given previous work on Ena/VASP showing that it modulates actin cytoskeleton organization, the Khc interaction was particularly interesting.

To determine if Khc and Ena bind one another in *Drosophila* cytosol, we tested for coimmunoprecipitation (Figure 2). First, cytosol was prepared from *Drosophila* S2 cells that had or had not been cotransfected with full-length Ena and Khc expression constructs. In nontransfected cells, anti-Ena antibodies precipitated Khc but anti-Khc antibodies did not precipitate detectable Ena, perhaps because of weaker antibody-antigen interaction. However, in overexpressing cells, each antibody clearly coprecipitated detectable amounts of both proteins (Figure 2A; homotypic staining is not published). To determine if Ena-Khc binding in cytosol was mediated by the region of Ena used in the yeast two-hybrid test, additional immunoprecipitations were done with S2 cells that expressed C-terminal truncations of Ena (Figure 2B). Expression of truncated Khc stalk/tail constructs was unsuccessful, so the reciprocal tests were not done. The coprecipitation results with truncated Ena indicated that amino acids 441–632 of Ena were required for Ena-Khc binding in cytosol. This segment includes parts of the proline-rich and EVH2 regions that contain known binding sites for Abl, profilin, actin, and Ena (see *Introduction*).

To determine if Ena and Khc bind one another in cytosol from intact neuronal tissue, immunoprecipitations were also performed with adult fly heads (Figure 2C). When anti-Khc antibodies were used, Ena coprecipitation was sometimes weak and sometimes not detected. However, anti-Ena antibodies consistently precipitated Khc. Considered together

Figure 2. Khc and Ena coimmunoprecipitate from *Drosophila* cytosol. (A) Western blots of unfractionated cytosol or immunoprecipitate (IP) fractions from normal S2 cells (lanes N) or from S2 cells cotransfected with full-length Ena and Khc expression vectors (lanes X). A blot of cytosol was stained first with anti-Khc R1.5 (top panel), then stripped and reprobed with anti-Ena-carboxy-terminal antibody (bottom panel). Separate blots of the immunoprecipitates (Ena-IP or Khc-IP) were stained with anti-Khc R1.5 (top panel) or anti-Ena-carboxy-terminal antibody (bottom panel). The immunoprecipitations were done with anti-Ena-carboxy-terminal antibody or anti-Khc Flyk2 antibody. (B) Western blots of unfractionated cytosol or anti-Khc Flyk2 immunoprecipitate (Khc-IP) from cells cotransfected with expression constructs for Khc and one of the following: full-length Ena (lanes 1), Ena lacking the C-terminal 52 amino acids (lanes 2), or Ena lacking the C-terminal 243 amino acids (lanes 3). Staining was done with an anti-Ena-amino-terminal antibody. Note that despite the abundance of truncated Ena, detectable amounts were not precipitated by the Khc antibody (lanes 3). This suggests a specific Khc binding site between amino acids 441 and 632 of Ena (see arrows in Figure 1A). (C) Western blot of various immunoprecipitates from adult fly-head cytosol stained with anti-Khc Flyk2. Immunoprecipitation was done with beads alone (mock-IP), anti-GFP (IgG-IP), anti-Ena-carboxy-terminal (Ena-IP), or anti-*Drosophila*-Khc from Cytoskeleton, (Khc-IP). The positions of prestained standards are noted beside the blots with numbers corresponding to their molecular weights. In all Khc- and Ena-IP experiments, either duplicate blots or stripped original blots were stained with the homotypic antibody to confirm robust precipitation of the primary protein (unpublished data).

with the observations with extracts from cultured cells, the coprecipitation from adult heads shows that Ena, a protein previously known for binding the actin cytoskeleton and influencing its organization, can also bind the microtubule motor protein kinesin-1 in *Drosophila*. This interaction and the fact that Khc, Abl, and Ena are neuronal proteins raised two questions: does Ena-Khc binding facilitate the anterograde transport of Ena into the axon, and/or does Ena have a kinesin-1 regulatory function?

Ena Does Not Behave Like a Fast-Axonal-Transport Cargo

To test the possibility that Ena is a fast-transport cargo of kinesin-1, we studied the effects of Khc mutations on Ena distribution in segmental nerves by immunostaining and fluorescence microscopy. Known fast-axonal-transport cargoes, such as vesicle-associated synaptic terminal proteins and mitochondria, accumulate in axonal swellings caused by mutation of kinesin-1 or dynein subunits (Hurd and Saxton, 1996; Gindhart *et al.*, 1998; Bowman *et al.*, 1999; Martin *et al.*, 1999). Slow-transport materials, such as tubulin, appear evenly distributed in the nerves of such motor mutants and do not accumulate in axonal swellings (Hurd and Saxton, 1996). Ena staining patterns in segmental nerves were compared with those of cysteine string protein (CSP), a known fast-transport component, in wild-type and partial-loss-of-function (hypomorphic) Khc mutant larvae (Figure 3). In wild-type larvae, CSP staining was faintly punctate in segmental nerves and strong in the boutons of motor-axon terminals (Figure 3, A and C). In Khc mutants, CSP accumulated to high levels in axonal swellings, consistent with its being a fast-axonal-transport cargo (Figure 3B). Ena did not accumulate in those swellings but instead maintained an even axonal distribution (Figure 3B). An obvious reduction in staining for both proteins in axon terminals (Figure 3D) was probably due to the substantial reduction in terminal size caused by Khc mutations (Hurd and Saxton, 1996). The lack of Ena accumulation in axonal swellings suggests that it is not carried by kinesin-1 as a fast-axonal-transport cargo. However, intermittent Ena-Khc interactions could facilitate Ena anterograde movement by slow axonal transport (Brown, 2003).

Genetic Tests Indicate Functional Interactions between Khc, Ena, and Abl

In a separate approach aimed at identifying proteins that have positive functional interactions with kinesin-1, a set of large chromosomal deletions was tested for "enhancer of Khc" (EK) activity by creating heterozygotes for each in a heterozygous Khc null background (*Khc*¹⁶/*Khc*⁺). That background does not itself cause defects, other than occasional small axonal swellings. However, when a heterozygous mutation in *Kinesin light chain* (*Klc*) is combined with a heterozygous Khc null, larvae develop numerous large axonal swellings and exhibit paralytic tail flipping (Martin *et al.*, 1999). Of 137 deletions that collectively uncovered ~75% of the *Drosophila* genome, 11 had EK activity (see Table S1 in Supplementary Materials). One of those deletions, *Df(3L)81k19*, removed a portion of the left arm of chromosome 3. To map the EK locus within that area, several smaller deletions were tested. Five with breakpoints in the 73A-74F polytene region had EK activity (Figure 4). The common deleted interval uncovered *Baldspot*, *Abl*, and three other predicted genes.

To identify the EK locus, single gene mutations were tested. Mutant *Baldspot* alleles did not have EK activity. However, two different hypomorphic alleles of *Abl* (*Abl*¹ and *Abl*⁴) did have EK activity, causing mild tail-flipping with a penetrance of 10–20% and a substantial enhancement of the size and abundance of axonal swellings (Figure 5). Heterozygous *Abl* alleles in a wild-type Khc background did not cause tail-flipping or swellings. Small deletions (*Df(3L)st-E36* and *Df(3L)st-j7*) that eliminate *Abl*, but not the neighboring *Abl* enhancer genes *Disabled* (*Dab*) and *Neurotactin*

Figure 3. Ena and CSP localization in wild-type and *Khc* mutant nerves and motor-axon terminals. Scanning confocal fluorescence micrographs of (A and B) larval segmental nerves and (C and D) motor-neuron terminals in segment A4 of wild-type (+/+) or *Khc*⁶/*Df*(2R)/*p6* mutant (*Khc*⁶/*Df*) third instar larvae. Staining was with mouse anti-CSP antibody and rabbit anti-Ena-amino-terminal antibody. The punctate background staining in the Ena panels of C and D is in muscle cells 6/7. Scale bar, 10 μ m.

(*Nrt*), caused an increase in the penetrance of tail flipping to 30% when heterozygous. Deletions that remove *Abl*, *Dab*, and *Nrt* together (*Df*(3L)81K19, *Df*(3L)*tra*, *Df*(3L)*st-b11*, and *Df*(3L)*std11*; Liebl *et al.*, 2000) increased tail-flipping penetrance to 50%, which was comparable to that seen in similar EK tests of a severe *Klc* mutation (Martin *et al.*, 1999). These results suggest that wild-type *Abl* and its interacting proteins *Dab* and/or *Nrt* have overlapping positive influences on the axonal transport activity of *Drosophila* kinesin-1.

To address the question of whether or not *Ena* influences the function of *Khc*, we tested for positive and negative genetic interactions. In animals that were *Khc*¹⁶ *ena*^{+/Khc} *ena*²¹⁰, no synthetic phenotypes were seen, suggesting that *Ena*-*Khc* binding does not have a positive influence on kinesin-1 function. The possibility of a direct negative *Ena* influence was tested by generating fly strains that carried a *Gal4:UAS-ena* overexpression transgene and various driver transgenes (*Act5C-GAL4*, *HSP70-GAL4*, or *elav-GAL4*). No axonal-transport phenotypes were seen, arguing against a negative influence on kinesin-1 by simple overexpression of *Ena*. However, in light of past overexpression studies and the likelihood that *Ena* activation is complex, a simple transgenic increase in *Ena* dosage may not be sufficient to generate an increase in *Ena* activity (Comer *et al.*, 1998). In a third test, we studied the effect of *ena* mutations on the synthetic *Abl*-*Khc* phenotypes. As opposed to the tail-flipping and abundant swellings seen in *Khc*¹⁶/*Khc*⁺; *Abl*¹/*Abl*⁺ double heterozygotes, triple heterozygotes (*Khc*¹⁶ *ena*^{+/Khc} *ena*²¹⁰; *Abl*¹/*Abl*⁺) showed no tail flipping and a marked reduction in axonal swellings (Figure 5). Thus, in a background sensitized by reducing the dose of *Khc* by one-half, axonal transport problems caused by reduced *Abl* activity can be prevented by also reducing *Ena* activity. This suggests that endogenous *Ena*, when not inhibited by *Abl*, has a negative influence on kinesin-1-driven axonal-transport.

Figure 4. Mapping a dominant enhancer of *Khc* to the *Abl* locus. Depicted at the top is a portion (bands 72E to 74A) of the left arm of chromosome 3 (from Bridges's polytene map; Lindsley and Zimm, 1992). On the left are the names of various deletions tested for EK activity in a *Khc*¹⁶/*Khc*⁺ genetic background. Black bars indicate the regions not deleted, while gaps indicate the deleted regions. Plus-symbols indicate definite EK activity. The genes *Abl*, *Baldspot* (*blp*), *Galpha73B*, *zetaCOP*, and *CG13032* are likely to be in the smallest deletion interval that has EK activity (73B01-02) based on sequence data (Flybase, 2005). Single gene mutant alleles of *Abl* and *Baldspot* were tested for EK activity. Mutant alleles of the other genes were not available (na).

Figure 5. Genetic interactions among *Khc*, *Abl*, and *ena*. Genotypes are noted in each panel: *Khc* = *Khc*¹⁶, *Abl* = *Abl*¹, and *ena* = *ena*²¹⁰. (A) Single video frames of third-instar larvae crawling on hard agar. Anterior is to the left. The arrow shows posterior paralysis or "tail flipping," a classic axonal-transport phenotype, in the *Khc Abl* double heterozygote. Larvae heterozygous for *Khc*¹⁶ alone (top) or *Abl*¹ alone (unpublished data) showed no tail flipping. (B) Immunofluorescence micrographs of third-instar larval segmental nerves (n) in abdominal segment A4 of larval neuromuscular preparations. Anti-synaptotagmin shows the locations of axonal swellings (e.g., arrow). These images are representative for each genotype, as determined by two observers scoring coded images. Larvae were ~3 mm in length. Scale bar for (B), 10 μ m.

Ena/VASP, when freed of negative regulation by Abl, facilitates the growth of abnormally long, unbranched actin filaments (Bear *et al.*, 2002). If the *Abl-Khc* synthetic interaction phenotypes reflect nonspecific changes in the cytoplasm, such as increased viscosity due to F-actin overgrowth, one would expect similar synthetic interactions between *Abl* and other axonal transport mutations. To test this, we looked for interactions between *Abl* and *Dhc64C*, which encodes the force-producing subunit of cytoplasmic dynein, a retrograde axonal transport motor. Homozygous hypomorphic *Dhc64C* mutations cause axonal swelling and tail-flipping phenotypes similar to those caused by *Khc* mutations (Martin *et al.*, 1999). Larvae doubly heterozygous for an *Abl* null and for *Dhc64C*^{ek1}, a null-like allele (Martin *et al.*, 1999), showed neither tail flipping nor axonal swelling enhancement. Thus, reduced Abl dosage does not cause a general hindrance of all fast-axonal-transport. This suggests that the Ena-Khc physical interaction and the *Abl-ena-Khc* genetic relationships reflect specific functional interactions.

DISCUSSION

We have identified functional relationships among the fast-antegrade-axonal-transport motor kinesin-1, the actin regulator Ena, and the nonreceptor tyrosine kinase Abl, which is a known negative regulator of Ena. Ena is present in axons and can bind kinesin-1, but immunostaining suggests that Ena is not a fast-axonal-transport cargo. Genetic interactions suggest that Abl has a positive influence on Khc, probably by inhibiting Ena. We suggest a model wherein Ena, when

Figure 6. A model for kinesin-1 regulation by Abl and Ena. Kinesin-1 is represented as a tetramer of two elongated Khc molecules (orange globular domains connected by black coiled-coil helix) plus two Klc molecules (gray crescents). It is shown linked to a cargo by unknown proteins (dark red ellipses). Activation pathways for Abl and Ena are not certain, as noted by white question marks. Inhibition effects are noted with a line and cross-bar. Activation effects are noted with a line and arrowhead.

not inhibited by Abl, can inhibit kinesin-1 cargo transport by interacting with the Khc stalk-tail (Figure 6). Hence, elevated Abl pathway activity, stimulated by extracellular and/or intracellular signals, inhibits Ena and enhances anterograde movement of kinesin-1 cargoes. Active Ena, by binding Khc, could trigger detachment of kinesin-1 from microtubules and release of its cargoes, which would make the cargoes available for short-range transport or anchorage by cytoplasmic myosins and the actin cytoskeleton (Tabb *et al.*, 1998).

The regions of Khc and Ena that interact are known to interact also with other proteins. The C-terminal coil-2 and tail portions of Khc include residues that participate in homo-dimerization, that can bind and inhibit the ATPase activity of its N-terminal motor domain, and that can bind Klc (reviewed by Verhey and Rapoport, 2001). Thus, Ena could inhibit transport by destabilizing Khc dimerization, by enhancing tail/motor domain interaction, or by disturbing Khc-Klc interactions. The C-terminal portion of Ena that binds Khc mediates Ena multimerization and binding of Abl, profilin, and other proteins (reviewed by Korey and Van Vactor, 2000; Krause *et al.*, 2003; Kwiatkowski *et al.*, 2003). One possibility raised by the three-way *Khc-Abl-ena* genetic interaction is that binding of activated Abl to Ena causes a conformational change that inhibits Ena binding to Khc. Tests of the effects of Abl overexpression on Ena-Khc interaction in cultured cells showed no consistent effect (Ahern-Djamali and Martin, unpublished results). However, as with tests of Ena overexpression (this report and Comer *et al.*, 1998), the activation and control of Abl and Ena may be too complex for this sort of approach to be informative.

The results of this study add to evidence that Abl influences both F-actin and microtubule cytoskeleton activities. Abl control of F-actin architecture and lamellipodial activity, through regulation of Ena/VASP proteins, is well known (see *Introduction*). It also has been demonstrated recently that Abl can regulate microtubule plus-end-polymerization dynamics at the periphery of neuronal growth cones by help-

ing transmit signals from Robo receptors to orbit, which is a microtubule plus-end-binding protein (Lee *et al.*, 2004). Our results, which suggest that Abl regulates a plus-end-directed microtubule motor protein, support the idea that Abl acts as a signaling “node” to coordinate the responses of F-actin and microtubule-based processes to extracellular signals.

Kinesin-1 has a variety of different cargoes that employ different linkage mechanisms (Verhey and Rapoport, 2001; Gunawardena and Goldstein, 2004). Thus, in addition to global kinesin-1 regulators, there may be specific regulators that influence the transport of specific subsets of cargoes. We suspect this is the case for Ena, because kinesin-1-driven anterograde transport of mitochondria appears normal in *Khc Abl* double-heterozygous mutant larval nerves (Bader, Pilling, and Saxton, unpublished results). Future studies of the transport of other axonal organelles should be informative. In addition to this issue of specificity, other key questions come to mind: Do transmembrane receptors like Robo use Abl and Ena to make axonal transport responsive to extracellular cues? Do Abl and Ena help coordinate changes in actin and growth cone dynamics with changes in fast-axonal-transport? Future experimentation that addresses questions about the Abl-Ena-Khc relationships should produce some interesting answers and may provide new insights into coordination between F-actin- and microtubule-based processes.

ACKNOWLEDGMENTS

We thank Allen Comer, Aaron Pilling, and Jason Bader for sharing unpublished results and Andrew Kastenmeier and Srinu Reddy for technical assistance. This work was supported by fellowships to M.A.M. from the Waltham Cancer Institute and the American Heart Association, Midwest Affiliate, as well as by National Institutes of Health Grants CA48582 (F.M.H.) and GM46295 (W.M.S.).

REFERENCES

- Ahern-Djamali, S. M., Bachmann, C., Hua, P., Reddy, S. K., Kastenmeier, A. S., Walter, U., and Hoffmann, F. M. (1999). Identification of profilin and src homology 3 domains as binding partners for *Drosophila* Enabled. *Proc. Natl. Acad. Sci. USA* 96, 4977–4982.
- Ahern-Djamali, S. M., Comer, A. R., Bachmann, C., Kastenmeier, A. S., Reddy, S. K., Beckerle, M. C., Walter, U., and Hoffmann, F. M. (1998). Mutations in *Drosophila* enabled and rescue by human vasodilator-stimulated phosphoprotein (VASP) indicate important functional roles for Ena/VASP homology domain 1 (EVH1) and EVH2 domains. *Mol. Biol. Cell* 9, 2157–2171.
- Bear, J. E. *et al.* (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* 109, 509–521.
- Bowman, A. B., Patel-King, R. S., Benashski, S. E., McCaffery, J. M., Goldstein, L.S.B., and King, S. M. (1999). *Drosophila* roadblock and *Chlamydomonas* LC7, a conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis. *J. Cell Biol.* 146, 165–179.
- Brown, A. (2003). Axonal transport of membranous and nonmembranous cargoes: a unified perspective. *J. Cell Biol.* 160, 817–821.
- Comer, A. R., Ahern-Djamali, S. M., Juang, J. L., Jackson, P. D., and Hoffmann, F. M. (1998). Phosphorylation of Enabled by the *Drosophila* Abelson tyrosine kinase regulates the in vivo function and protein-protein interactions of Enabled. *Mol. Cell. Biol.* 18, 152–160.
- Flybase. (2005). A genome database. <http://flybase.bio.indiana.edu/>.
- Gertler, F. B., Bennett, R. L., Clark, M. J., and Hoffmann, F. M. (1989). *Drosophila* Abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with Disabled. *Cell* 58, 103–113.
- Gertler, F. B., Comer, A. R., Juang, J. L., Ahern, S. M., Clark, M. J., Liebl, E. C., and Hoffmann, F. M. (1995). *enabled*, a dosage-sensitive suppressor of mutations in the *Drosophila* Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties. *Genes Dev.* 9, 521–533.
- Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996). Mena, a relative of VASP and *Drosophila* Enabled, is implicated in the control of microfilament dynamics. *Cell* 87, 227–239.
- Gindhart, J. G., Desai, C. J., Beushausen, S., Zinn, K., and Goldstein, L.S.B. (1998). Kinesin light chains are essential for axonal transport in *Drosophila*. *J. Cell Biol.* 141, 443–454.
- Grevengoed, E. E., Fox, D. T., Gates, J., and Peifer, M. (2003). Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J. Cell Biol.* 163, 1267–1279.
- Grevengoed, E. E., Loureiro, J. J., Jesse, T. L., and Peifer, M. (2001). Abelson kinase regulates epithelial morphogenesis in *Drosophila*. *J. Cell Biol.* 155, 1185–1198.
- Gunawardena, S., and Goldstein, L.S.B. (2004). Cargo-carrying motor vehicles on the neuronal highway: transport pathways and neurodegenerative disease. *J. Neurobiol.* 58, 258–271.
- Hernandez, S. E., Krishnaswami, M., Miller, A. L., and Koleske, A. J. (2004). How do Abl family kinases regulate cell shape and movement? *Trends Cell Biol.* 14, 36–44.
- Hurd, D. D., and Saxton, W. M. (1996). Kinesin mutations cause motor neuron disease phenotypes by disrupting fast axonal transport in *Drosophila*. *Genetics* 144, 1075–1085.
- Korey, C. A., and Van Vactor, D. (2000). From the growth cone surface to the cytoskeleton: one journey, many paths. *J. Neurobiol.* 44, 184–193.
- Krause, M., Dent, E. W., Bear, J. E., Loureiro, J. J., and Gertler, F. B. (2003). Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu. Rev. Cell Dev. Biol.* 19, 541–564.
- Kwiatkowski, A. V., Gertler, F. B., and Loureiro, J. J. (2003). Function and regulation of Ena/VASP proteins. *Trends Cell Biol.* 13, 386–392.
- Lee, H., Engel, U., Rusch, J., Scherrer, S., Sheard, K., and Van Vactor, D. (2004). The microtubule plus end tracking protein Orbit/MAST/CLASP acts downstream of the tyrosine kinase Abl in mediating axon guidance. *Neuron* 42, 913–926.
- Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M., and Seeger, M. A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF *trio* reveal *trio*'s role in axon pathfinding. *Neuron* 26, 107–118.
- Lindsley, D., and Zimm, G. (1992). *The Genome of Drosophila melanogaster*, San Diego: Academic Press.
- Littleton, J. T., Bellen, H. J., and Perin, M. S. (1993). Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. *Development* 118, 1077–1088.
- Martin, M. A., Iyadurai, S. J., Gassman, A., Gindhart, J. G., Hays, T. S., and Saxton, W. M. (1999). Cytoplasmic dynein, the dynactin complex and kinesin are interdependent and essential for fast axonal transport. *Mol. Biol. Cell* 10, 3717–3728.
- Miller, A. L., Wang, Y., Mooseker, M. S., and Koleske, A. J. (2004). The Abl-related gene (Arg) requires its F-actin-microtubule cross-linking activity to regulate lamellipodial dynamics during fibroblast adhesion. *J. Cell Biol.* 165, 407–419.
- Saxton, W. M., Hicks, J., Goldstein, L.S.B., and Raff, E. C. (1991). Kinesin heavy chain is essential for viability and neuromuscular functions in *Drosophila*, but mutants show no defects in mitosis. *Cell* 64, 1093–1102.
- Saxton, W. M., Porter, M. E., Cohn, S. A., Scholey, J. M., Raff, E. C., and McIntosh, J. R. (1988). *Drosophila* kinesin: characterization of microtubule motility and ATPase. *Proc. Natl. Acad. Sci. USA* 85, 1109–1113.
- Tabb, J. S., Molyneaux, B. J., Cohen, D. L., Kuznetsov, S. A., and Langford, G. M. (1998). Transport of ER vesicles on actin filaments in neurons by myosin V. *J. Cell Sci.* 111, 3221–3234.
- Vale, R. D. (2003). The molecular motor toolbox for intracellular transport. *Cell* 112, 467–480.
- Verhey, K. J., and Rapoport, T. A. (2001). Kinesin carries the signal. *Trends Biochem. Sci.* 26, 545–550.
- Woodring, P. J., Hunter, T., and Wang, J. Y. (2003). Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. *J. Cell Sci.* 116, 2613–2626.
- Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N., and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science* 263, 977–980.