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The Tyrosine Kinase Abl and Its Substrate Enabled Collaborate with the Receptor Phosphatase Dlar to Control Motor Axon Guidance

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Summary

Genetic analysis of growth cone guidance choice points in *Drosophila* identified neuronal receptor protein tyrosine phosphatases (RPTPs) as key determinants of axon pathfinding behavior. We now demonstrate that the *Drosophila* Abl tyrosine kinase functions in the intersegmental nerve b (ISNb) motor choice point pathway as an antagonist of the RPTP Dlar. The function of Abl in this pathway is dependent on an intact catalytic domain. We also show that the Abl phosphoprotein substrate Enabled (Ena) is required for choice point navigation. Both Abl and Ena proteins associate with the Dlar cytoplasmic domain and serve as substrates for Dlar in vitro, suggesting that they play a direct role in the Dlar pathway. These data suggest that Dlar, Abl, and Ena define a phosphorylation state–dependent switch that controls growth cone behavior by transmitting signals at the cell surface to the actin cytoskeleton.

Introduction

To reach correct targets in the developing nervous system, axons must navigate through a complex embryonic landscape. From seminal experiments in a variety of systems (e.g., Lance-Jones and Landmesser, 1981; Bentley and Caudy, 1983; Bastiani et al., 1984; Kuwada, 1986), we know that neuronal growth cones depend on specific guidance information presented along the path to their final destinations. Recent progress has identified some attractive and repellent extracellular factors that convey guidance information, as well as neuronal receptor molecules that receive these different inputs (reviewed by Tessier-Lavigne and Goodman, 1996). However, we understand far less about the mechanisms that interpret this information and convert extracellular cues into directional cell motility.

Directed axon growth appears to result from rapid remodeling of the cytoskeleton just beneath the cell surface (e.g., Letourneau and Marsh, 1984; Bentley and Toroian-Raymond, 1986). Faced with a complex environment, growth cones weigh a balance of positive and negative factors to make correct decisions. Attractive cues appear to foster local actin assembly (Lin and Forscher, 1993; O'Connor and Bentley, 1993), whereas repellent cues stimulate the collapse of actin structures (Fan and Raper, 1995). While the direct links between cell surface receptors and the actin-based motility machinery are still unclear, accumulated evidence points to tyrosine phosphorylation as a key means of transducing and integrating guidance information. Within the growth cone, phosphotyrosine is enriched at the tips of filopodia and is modulated by factors that influence axon outgrowth (Wu and Goldberg, 1993). Moreover, inhibitors that block tyrosine kinases or phosphatases have dramatic effects on axon extension in vitro (Bixby and Jhabvala, 1992; Goldberg and Wu, 1995) and can alter axon pathfinding behavior in vivo (Menon and Zinn, 1998). However, more compelling evidence comes from the structures of guidance molecules themselves.

Some axon guidance receptors contain protein kinase domains, such as the Eph receptor tyrosine kinases (RTKs) (reviewed by Flanagan and Vanderhaegen, 1998) and *Drosophila* Derailed (Callahan et al., 1995). Other transmembrane molecules that affect guidance decisions contain protein tyrosine phosphatase domains (RPTPs), such as *Drosophila* Dlar, DPTP69D, and DPTP99A (reviewed by Desai et al., 1997b; Stoker and Dutta, 1998; Van Vactor, 1998a). In addition, the outgrowth-promoting activities of several axonal cell adhesion molecules (CAMs) appear to depend on different tyrosine kinases, including Src, Fyn, and the basic fibroblast growth factor receptor (reviewed by Van Vactor, 1998b). Moreover, protein phosphatase activity can be induced by antibodies that cross-link L1 and NCAM (Klinz et al., 1995). These observations have implicated tyrosine phosphorylation in the downstream response to axon guidance cues. However, previous analysis of kinase-inactivating mutations in EphB2 (Nuk) and *Drosophila* Abl suggests that kinase activity may not always be required for function in vivo (Henkemeyer et al., 1990, 1996; Hill et al., 1995; Orioli et al., 1996).

Analyses of the signaling pathways associated with some protein tyrosine kinases (PTKs) suggest downstream links to cytoskeleton. *Drosophila* Abl (d-Abl), an intracellular PTK expressed in developing axons, is a good example. Dose-sensitive genetic screens for loci that interact with d-Abl have identified several genes important in axonal development, including *disabled* (*dab*; Gertler et al., 1989, 1993), *fax* (Hill et al., 1995), and the Abl phosphoprotein substrate *enabled* (*ena*; Gertler et al., 1990, 1995). Ena is a member of a protein family conserved from fly to human, including mammalian Ena (Mena), Evl, and vasodilator-stimulated phosphoprotein (VASP) (Gertler et al., 1996). Interestingly, Mena, Evl, and VASP are recruited by the bacterial protein ActA to the site of actin assembly required for the motility of the intracellular pathogen Listeria monocytogenes (Pistor et al., 1995; Gertler et al., 1996; Niebuhr et al., 1997). In this model system for actin-based motility, disruption of the interaction between ActA and VASP

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has been shown to impair Listeria motility and pathogenicity (Smith et al., 1996; Niebuhr et al., 1997). Ena/VASP family members bind to the actin regulatory protein Profilin (Reinhard et al., 1995; Gertler et al., 1996; F. Gertler, personal communication), which has also been implicated in Listeria motility (Theriot et al., 1994). These observations, coupled with the fact that overexpression of a Mena isoform in fibroblasts induces actin-rich cellular protrusions (Gertler et al., 1996), support the conclusion that members of the Ena family control the cytoskeletal motility apparatus.

In part, it has been difficult to understand the precise role of Ena and Abl in *Drosophila* axon guidance, because neither protein has been shown to associate or function directly with a specific neuronal cell surface receptor, despite a number of intriguing genetic interactions. In addition, most previous characterizations of *abl* pathway phenotypes employed general axon markers (e.g., mAb BP102) that make analysis of specific guidance choices difficult. Embryos lacking Abl and other genes (e.g., *dab*, *fas I*, *fax*, *prospero*, *armadillo* and *notch*) were shown to display gross defects in many axon pathways (Gertler et al., 1989, 1995; Elkins et al., 1990; Hill et al., 1995; Giniger, 1998; Loureiro and Peifer, 1998). However, axon defects were not observed when *abl* alone was absent (Gertler et al., 1989; Elkins et al., 1990). This led to speculation that d-Abl function is in some way redundant with other signaling molecules (Hoffmann, 1991). However, in a companion paper, we show that *abl* function is nonredundant for aspects of intersegmental nerve b (ISNb) motor axon development (Wills et al., 1999 [this issue of *Neuron*]).

Genetic analysis of motor axon choice points in the *Drosophila* embryo has revealed a number of components required for the correct guidance of ISNb growth cones (e.g., Sink et al., 1993, Soc. Neurosci., abstract; Van Vactor et al., 1993; Desai et al., 1996; Fambrough and Goodman, 1996; Krueger et al., 1996; Korey et al., 1997, Soc. Neurosci., abstract; Kaufmann et al., 1998; Winberg et al., 1998; Yu et al., 1998). The discovery that three of these genes are members of the receptor-like protein tyrosine phosphatase (RPTP) family suggests that regulated tyrosine phosphorylation functions to guide motor growth cones in this context. Since RPTP catalysis presupposes the activity of some PTK, the requirement for Abl function in neurons that also require Dlar raised the question of whether Abl function might be linked to the cell surface through some relationship with Dlar. Thus, we examined genetic and biochemical interactions between Abl and Dlar. We describe here the discovery that Dlar function at the ventral motor choice point is highly sensitive to Abl activity and that Abl associates directly with the Dlar cytoplasmic domain. Our analysis of loss- and gain-of-function *abl* backgrounds provides compelling evidence that Abl kinase activity is required for its role in choice point navigation. Moreover, we show not only that Ena is required at the same choice point but also that Ena can associate directly with Dlar. Abl, Ena, and Dlar appear to participate in a phosphorylation-dependent switch that connects the cell surface to the actin-based machinery responsible for directional cell motility.

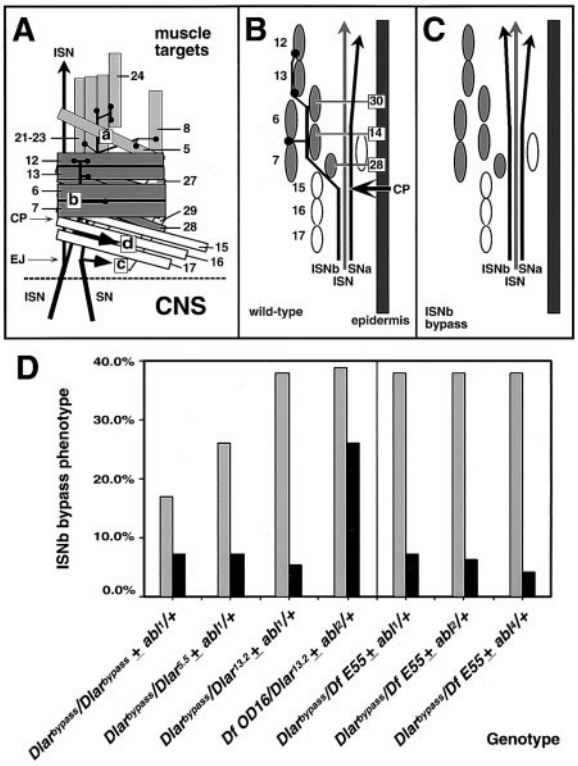

Figure 1. Genetic Interactions between the Dlar Phosphatase and the Abl Kinase

(A) The motor axon projections of ISNb are shown schematically as viewed in a flat fillet preparation and in cross section to identify the anatomical features in the ventral target domain of a stage 16–17 wild-type *Drosophila* embryo (in abdominal segments A2–A7). The ISNb bypass phenotype is shown in cross-sectional view, where ISNb axons fail to turn at contacts with muscles 14 and 28, and instead follow the trajectory of ISN axons toward inappropriate dorsal targets.

(B) The frequencies of ISNb bypass phenotypes in different *Dlar* mutant backgrounds (gray bars) are compared with the same *Dlar* mutations in the presence of a single allele of *abl* (black bars). The number of embryonic hemisegments (abdominal segments A2–A7) scored with mAb1D4 at stage 17 is shown in parentheses for each genotype. $Dlar^{bypass}/Dlar^{bypass} \pm abl^{2}/+$ (n = 257, n = 185) (1); $Dlar^{bypass}/Dlar^{5.5} \pm abl^{2}/+$ (n = 187, n = 175) (2); $Dlar^{bypass}/Dlar^{13.2} \pm abl^{2}/+$ (n = 172, n = 340) (3); $Dlar^{13.2}/Df(3L)OD16 \pm abl^{1}/+$ (n = 166, n = 149) (4); $Dlar^{bypass}/DF(3L)E55 \pm abl^{1}/+$ (n = 248, n = 145) (5); $Dlar^{bypass}/DF(3L)E55 \pm abl^{2}/+$ (n = 248, n = 268) (6); $Dlar^{bypass}/DF(3L)E55 \pm abl^{4}/+$ (n = 248, n = 277) (7); and $Dlar^{bypass}/DF(3L)E55 \pm src64^{\Delta 17},e/+$ (n = 248, n = 162) (8).

Results

Drosophila Abl Is a Potent Suppressor of the Dlar Phenotype

Dose-dependent genetic interaction has been widely used to identify gene products that function in a particular signaling pathway (e.g., Gertler et al., 1990; Simon et al., 1991). The reciprocal catalytic activities of a tyrosine kinase and phosphatase predict that a reduction in kinase activity within the Dlar pathway might suppress the *Dlar* motor axon phenotype. In *Dlar* mutant embryos, subsets of axons derived from the intersegmental nerve route (ISN), called ISNb and ISNd (Figures 1A and 1B), fail to enter adjacent muscle target domains just outside

the ventral nerve cord (Krueger et al., 1996). Instead, *Dlar* mutant ISNb and ISNd axons follow the ISN toward dorsal targets (the "bypass" phenotype; see schematic in Figure 1C). Our recent observation that *abl* loss of function disrupts the outgrowth of ISNb (Wills et al., 1999) made the Abl tyrosine kinase an excellent candidate for a role in *Dlar* signaling. Therefore, we examined various genetic backgrounds in which homozygous *Dlar* mutations were combined with mutations in a single allele of *abl*. Reduction of *abl* up to half of normal gene dose had a profound effect on the penetrance of the *Dlar* motor axon guidance phenotype, suppressing the *Dlar* phenotype up to 10-fold, as assessed with the anti-Fasciclin II antibody 1D4 (Van Vactor et al., 1993; see Experimental Procedures); for example, ISNb bypass in $Dlar^{bypass}/DF(3L)E55$ is reduced from 38% to 4% with the addition of $abl^4/+$ (Figure 1D).

Suppression of ISNb bypass was independent of the specific *abl* or *Dlar* alleles used (including the Dlar alleles $Dlar^{bypass}$, $Dlar^{5.5}$, $Dlar^{13.2}$, *Df(3L)OD16*, and *DF(3L)E55* and *abl* alleles abl^1, abl^2, and abl^4). Reduction of *abl* also suppressed the ISNd phenotype in *Dlar* mutants, indicating that Abl function is not ISNb-specific; ISNd defects in a $Dlar^{bypass}/DF(3L)E55$ background drop from 53% (n = 140) to 19% (n = 108) when a single copy of abl^1 is introduced. It is interesting that reduced *abl* suppressed a *Dlar* zygotic null background ($DfOD16/Dlar^{13.2}$) to a lesser degree than the hypomorphic combinations (Figure 1D). This difference is likely to reflect the fact that the $Dlar^{bypass}$ allele encodes a protein that retains the D1 PTP domain (D. Scalice and D. V. V., unpublished data).

Abl Binds Directly to the Dlar Cytoplasmic Domain

Although genetic interactions provide a powerful tool for detecting functional relationships between gene products in vivo, a mechanistic understanding of the biochemical events in a signaling pathway depends on knowing whether interactions are direct or indirect. For example, genetic interactions exist between Abl and several putative guidance molecules, but direct physical interactions have been demonstrated only in the case of Ena (Gertler et al., 1995; Comer et al., 1998). Thus, fusion proteins were constructed that link glutathione S-transferase (GST) to one or both of the cytoplasmic PTP domains of Dlar or the PTP domains of two other *Drosophila* phosphatases, DPTP69D and DPTP10D (Figure 2D). These GST–PTP fusion proteins were then exposed to extracts made from *Drosophila* Schneider (S2) cells known to express Abl protein. Western blot analysis of GST "pulldowns" from extracts shows that endogenous Abl protein binds specifically to the full-length Dlar cytoplasmic domain (GST–Dlar D1–D2; Figure 2A). Neither GST alone nor GST fusions to other PTP domains shows a detectable association with Abl under these conditions (Figure 2A). As the concentration of extract is increased, Abl binds to GST–Dlar D2 alone but not significantly with wild-type D1 alone (data not shown).

The association of Dlar and Abl in S2 extracts is consistent with a direct functional relationship between the two proteins. However, the binding could depend on other factors present in the crude extract. Therefore, we examined the association of purified recombinant Abl protein with Dlar fusion proteins in the absence of other *Drosophila* proteins. Recombinant d-Abl binds to Dlar with somewhat less specificity as the d-Abl endogenous to S2 cells, binding to GST–Dlar D1–D2 and to GST–Dlar D2 but not significantly to GST–Dlar D1 (Figure 2B). In addition, we discovered that purified v-Abl binds to Dlar under the same conditions, with a profile of specificity very similar to that of d-Abl (Figure 2C). Since v-Abl represents only the kinase and SH2 domains of Abl, these domains appear sufficient to mediate Dlar binding. As further evidence of direct physical interactions between Abl and the Dlar D2 domain, kinase assays reveal that d-Abl phosphorylates GST–Dlar D2 in vitro (Figure 2E); the same specificity is seen for recombinant v-Abl (Figure 2F). In addition to the Dlar D2 domain, d-Abl can weakly phosphorylate the D2 domain of DPTP69D (Figure 2E); this is interesting, given reports that DPTP69D is tyrosine phosphorylated in S2 cells (Fashena and Zinn, 1997). Very low levels of tyrosine phosphorylation can be detected when d-Abl is exposed to a catalytically inactive C-to-S mutant form of Dlar D1 (see Experimental Procedures), suggesting that d-Abl can interact weakly with D1 and that Dlar D1 can dephosphorylate itself (data not shown). The physical interactions between Abl and Dlar support a model whereby both proteins function in the same signaling pathway. Furthermore, the phosphorylation of the D2 domain in vitro raises the intriguing possibility that d-Abl activity regulates Dlar function in vivo.

Abl Gain of Function Mimics Dlar Loss of Function

The contrast between the *abl* and *Dlar* phenotypes and the suppression of the *Dlar* phenotype by *abl* alleles suggest that Abl and Dlar play functionally antagonistic roles in ISNb development. This hypothesis makes a simple prediction: gain of function in Abl should result in a phenotype similar to loss of Dlar. Therefore, we used the GAL4 expression system (Brand and Perrimon, 1993) to target high-level expression of wild-type Abl to postmitotic neurons and then examined the development of motor axon pathways. With three independent neural specific GAL4 drivers (P[C155-GAL4], P[1407-GAL4], and P[elav-GAL4]), in combination with an *abl* cDNA under the control of the GAL4 upstream activator sequence (UAS), we observe consistent, GAL4-dependent phenotypes. Using Western blot analysis, we confirmed that Abl protein is overexpressed in neural-GAL4;UAS-abl^+ and neural-GAL4;UAS-$abl^{(K-N)}$ embryos (data not shown). When wild-type Abl is overexpressed, ISNb axons bypass their ventral target muscles in a manner indistinguishable from that of the ISNb phenotype observed in *Dlar* mutants (Figures 3B and 3D). These phenotypes were not observed in embryos carrying the GAL4 drivers alone or the UAS-abl^+ construct alone (Figure 3D). Furthermore, the frequency of ISNb bypass in each GAL4-UAS-abl^+ genotype correlates with the strength of the GAL4 driver as previously observed (Kaufmann et al., 1998), suggesting a dose-dependent relationship. The strongest ISNb bypass phenotype achieved with P[elav-GAL4];P[UAS-*abl*] (31%, n =

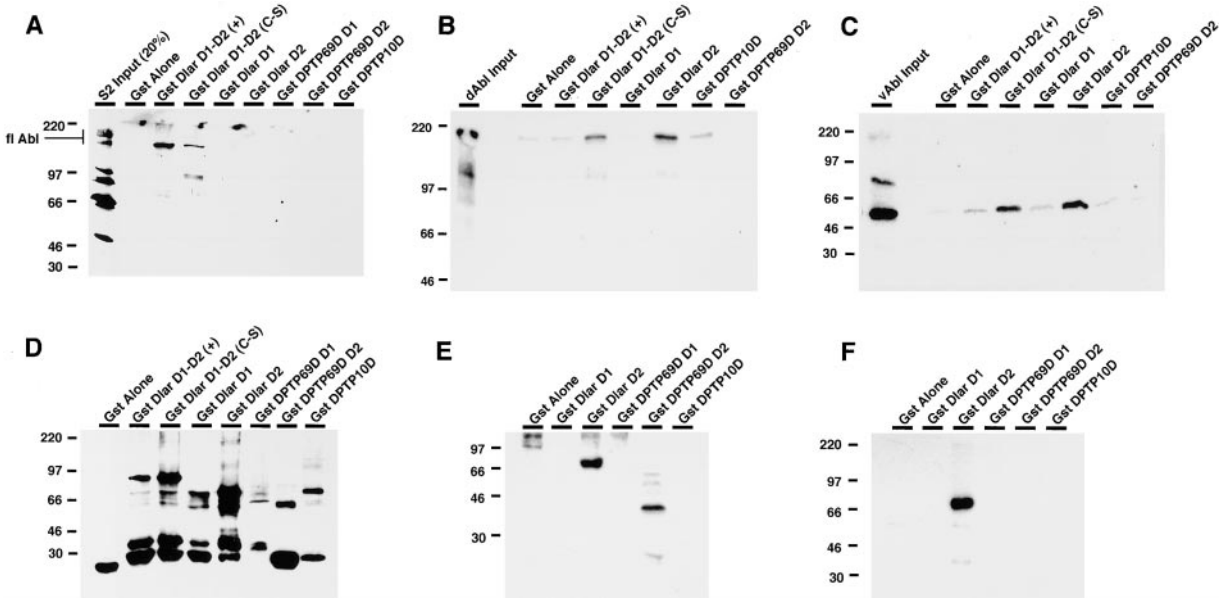

Figure 2. Physical Interactions between the Dlar Phosphatase and the Abl Kinase

(A) Endogenous Abl protein expressed in S2 cell lysates associates specifically with GST fusion proteins that contain the cytoplasmic domain of Dlar, as visualized by Western blot with anti-Abl antibodies following SDS–PAGE. Lane 1 contains 20% of the crude cell lysate applied to equal amounts of the GST fusion proteins in lanes 2–9. Full-length isoforms of Abl are indicated; degradation products and a couple of nonspecific bands are also seen at lower molecular weights. The fusion proteins in each lane are indicated above the gel. Molecular weight markers are shown in kilodaltons.

(B and C) Purified, recombinant *Drosophila* Abl (B) or v-Abl (C) binds to GST–Dlar in vitro, as assessed by a pull down assay in which dAbl protein has been prelabeled with γ^{32}P-ATP before mixing with the fusion proteins. The Abl input to the pull down assay is shown in the first lane of each gel.

(D) The various fusion proteins used in (A) through (C) are shown as visualized on a Western blot with anit-GST antibodies. Note that in addition to the full-length forms of each fusion protein, there is some accumulation of degradation products near the size of GST alone (seen in first lane).

(E) The phosphorylation of GST fusion proteins by purified d-Abl is shown following a kinase assay with γ^{32}P-ATP (see Experimental Procedures) followed by autoradiography.

(F) The phosphorylation of GST fusion proteins by v-Abl is shown as performed in (E).

186) approaches the penetrance of a strong loss of Dlar activity. To be certain that the ISNb bypass phenotypes caused by Abl misexpression did not result from a change in the fates or early pathfinding decisions of ISNb neurons, we used the anti-Fasciclin III antibody 2D5 to specifically stain the soma and early axon trajectories of RP1, RP3, and RP4 in P[elav-GAL4];P[UAS-*abl*] embryos; no defects above background were observed (1% defects in n = 337 hemisegments at embryonic stage 16). Although some misexpression phenotypes in *Drosophila* disappear during larval development (e.g., Lin and Goodman, 1994), the failure of ISNb innervation in Abl gain-of-function genotypes persists until late third instar (J. Wang et al., unpublished data).

To explore the specificity of the Abl gain-of-function phenotype, we also misexpressed wild-type Dsrc64 under control of GAL4. Only background levels of ISNb bypass phenotypes were observed when P[UAS-*Dsrc64#G7*] was crossed to the strongest neural driver, P[elav-GAL4] (Figure 3D). Interestingly, wild-type Dsrc64 misexpression does result in an ISNb stop short phenotype within the ventral muscle domain (56%, n = 219); this phenotype is reminiscent of *abl* loss of function (Wills et al., 1999). However, preliminary data suggest that neural overexpression of a truncated form of Src lacking the C-terminal regulatory domain does yield an ISNb bypass phenotype (J. Bateman et al., unpublished data), suggesting a regulatory difference between Abl and Src signaling in motor growth cones. For further evidence of specificity in the Abl gain-of-function phenotype, we misexpressed other genes in the Abl pathway that were originally identified as genetic enhancers of *abl* mutant phenotypes (Gertler et al., 1989; Hill et al., 1995). No ISNb bypass phenotypes were observed when *disabled* (*dab*) was expressed under the control of P[elav-GAL4] (0% ISNb, n = 205 embryonic stage 16–17 A2–A7 hemisegments). Likewise, no ISNb bypass phenotypes were observed in embryos that misexpress Fax (N. Sheard et al., unpublished data).

Although the Abl misexpression phenotype matched our prediction based on loss-of-function interactions, it is sometimes difficult to know whether a gain of function acts via the normal pathway. We reasoned that if the Abl gain-of-function effect is manifest through the same pathway as Dlar, then overexpression of Dlar should suppress this effect. Therefore, we expressed a P[UAS-*Dlar*$^{+}$] transgene previously shown to rescue the *Dlar* ISNb phenotype (Krueger et al., 1996) in combination with P[UAS-*abl*$^{+}$] under the control of P[elav-GAL4]. This coexpression attenuated the ISNb bypass phenotype to 13% (n = 257 embryonic stage 16–17 A2–A7 hemisegments), consistent with the notion that Abl displays a

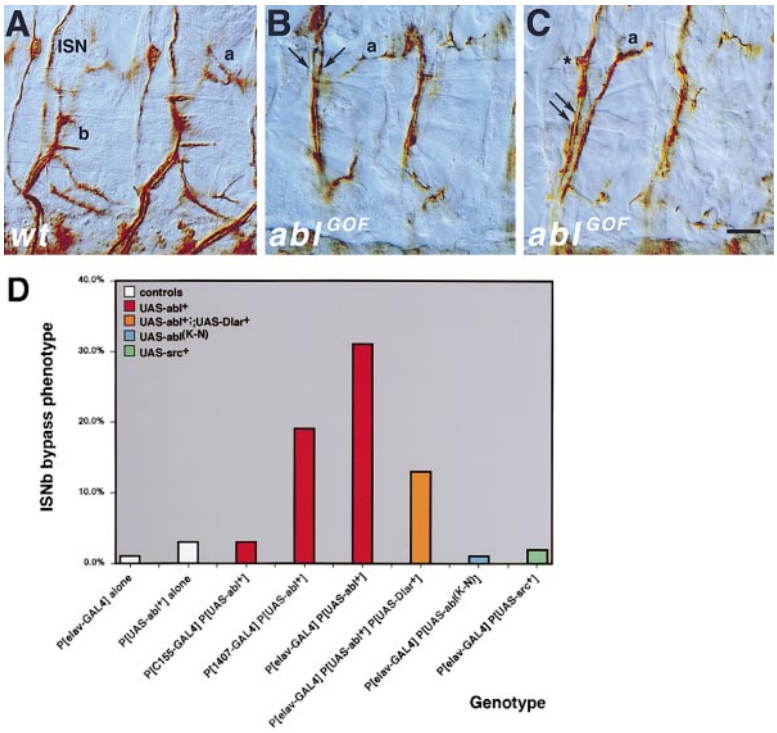

Figure 3. Abl Misexpression in Postmitotic Neurons Generates a Kinase-Dependent ISNb Bypass Phenotype

(A) Wild-type motor axon pathways are shown in an embryonic stage 17 fillet stained with mAb1D4. The intersegmental nerve (ISN), ISNb (b), and SNa (a) are labeled.

(B and C) In embryos that express the P[UAS-*abl*$^{+}$] transgene under control of the neural specific driver, P[elav-GAL4], ISNb axons extend beyond the ventral longitudinal muscle targets. Although SNa axons project normally to lateral targets (a), ISNb axons can be seen as a distinct fascicle following the ISN toward dorsal muscles (see arrows). Occasionally, misguided ISNb axons will extend a contact to ventral muscle 12 once past the target domain (asterisk).

(D) Quantitation of ISNb phenotypes is assessed with mAb1D4 in different misexpression genotypes in which UAS transgenes are placed under the control of neural specific GAL4 drivers. Negative controls are shown in white, Abl misexpression in dark gray, Abl kinase dead misexpression in light gray, and Src misexpression in black. P[elav-GAL4] alone (n = 178 stage 17 abdominal hemisegments A2–A7), levels of background phenotype are comparable in P[C155-GAL4] and P[1407-GAL4] (1); P[UAS-*abl*$^{+}$] alone (n = 249) (2); P[C155-GAL4];P[UAS-*abl*$^{+}$] (n = 266) (3); P[1407-GAL4];P[UAS-*abl*$^{+}$] (n = 297) (4); P[elav-GAL4];P[UAS-*abl*$^{+}$] (n = 186) (5); P[elav-GAL4];P[UAS-*abl*$^{+}$];P[UAS-*Dlar*$^{+}$] (n = 257) (6); P[elav-GAL4];P[UAS-*abl*$^{(K-N)}$] (n = 317) (7); and P[elav-GAL4];P[UAS-*Dsrc64*$^{+}$] (n = 219) (8).

Scale bar, ~10 μm.

gain-of-function phenotype by overwhelming the endogenous phosphatase.

Abl Function in ISNb Axons Is Dependent on an Active Kinase Domain

The antagonistic relationship between Abl and Dlar during ISNb development suggests a mechanism whereby both proteins regulate the phosphorylation of a common set of substrates. Such a model predicts that the kinase activity of Abl is necessary for its role in ISNb neurons. In a companion paper, we demonstrate that a kinase-inactivated Abl transgene previously shown to attenuate certain *abl* phenotypes (Henkemeyer et al., 1987, 1990) fails to rescue the ISNb axon phenotype of *abl* mutants (Wills et al., 1999). The ISNb bypass phenotype observed in embryos that overexpress wild-type Abl gave us the opportunity to ask the reciprocal question: is Abl kinase activity responsible for the gain-of-function phenotype? In contrast to wild-type Abl, when the K-to-N mutant form of Abl was expressed under control of the strongest neuronal GAL4 driver (P[elav-GAL4]), the frequency of ISNb bypass phenotypes did not significantly exceed background (Figure 3D). The strict requirement for an active kinase domain in both loss- and gain-of-function ISNb phenotypes suggests that the phosphorylation state of an Abl substrate plays a central role in choice point navigation.

Enabled Is Required for Choice Point Axon Guidance

Evidence for direct interaction between Abl and pathway components identified in *Drosophila* is strongest for Ena. Since Ena acts as a genetic antagonist of Abl (Gertler et al., 1990), we reasoned that loss of Ena should resemble gain of Abl. Previous analysis of *ena* mutants that demonstrated gross defects in embryonic axon pathways (Gertler et al., 1995) did not address axon guidance at specific choice points. Our analysis using the 1D4 antibody as a probe revealed ISNb bypass phenotypes in all *ena* mutant combinations (Figure 4). The penetrance of bypass was 86% in *ena*^{GC1}/*ena*^{GC5} (n = 185 embryonic stage 16 A2–A7 hemisegments) and 29% in *ena*^{GC5}/*ena*^{GC8} (n = 196; *ena*^{GC8} appears to be hypomorphic for motor axon guidance). Two types of ISNb phenotypes are observed in *ena* mutants: first, failure of ISNb to enter the ventral muscles after a successful defasciculation (characteristic of embryos lacking Dlar alone; Krueger et al., 1996) and second, failure of ISNb axons to defasciculate from the ISN pathway (characteristic of embryos lacking multiple phosphatases; Desai et al., 1996). In addition, the frequency of ISNb bypass in strong *ena* mutants is twice that observed in the strongest *Dlar* alleles. These observations may indicate that Ena acts as a point of convergence for multiple inputs in the ISNb guidance mechanism.

To confirm that the *ena* ISNb phenotypes in the muscle

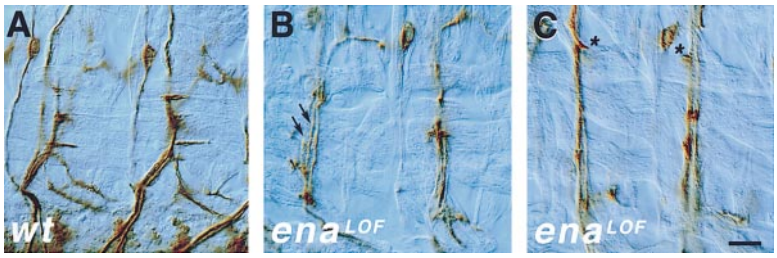

Figure 4. *enabled* Mutants Display the ISNb Bypass Phenotype

(A) Wild-type motor pathway anatomy is shown as seen in Figure 3A.

(B) In ena^{GC5}/ena^{GC8} embryos, ISNb axons extend beyond the ventral longitudinal muscles as a distinct fascicle parallel to the ISN (see arrows). SNa axons also follow an abnormal trajectory in these embryos, making an anterior turn toward lateral target muscles only after they extend beyond the longitudinal muscles.

(C) A second class of bypass phenotype is observed in ena^{GC5}/ena^{GC8} embryos, in which ISNb axons fail to defasciculate from ISN axons. Once past their targets, ISNb axons often reach back to contact ventral muscle 12 (see asterisks).

Scale bar, ~10 μm.

field are not secondary to pathfinding or cell fate defects within the ventral nerve cord, we also used the 2D5 (anti-FasIII) antibody to characterize the soma and early trajectories of RP1, RP3, and RP4 in *ena* mutants; although somal position and initial axon polarity were normal in *ena* mutants, indicating that RP cell fate was assigned correctly, a few RP axon pathfinding defects were observed within the ventral nerve cord. In ena^{GC5}/ena^{GC8} embryos, a small number of RP axons followed an aberrant path within the CNS (~11%; see Experimental Procedures) but usually found an ISN exit point to reach the periphery; this penetrance of early axonal defects is insufficient to account for the 29% frequency of ISNb bypass in this background. Thus, among functional requirements in a variety of guidance decisions, Ena is necessary for correct navigation of the ventral choice point.

Ena family members share a conserved domain structure, including an N-terminal EVH1 domain that mediates binding to Zyxin and Listeria ActA, a proline-rich region that supports associations with Profilin and SH3 domains, and a C-terminal EVH2 domain that promotes multimerization (Gertler et al., 1996). Mutations are available that specifically disrupt either the EVH1 or the EVH2 domains of Ena (ena^{210} and ena^{23}, respectively; Ahern-Djamali et al., 1998); both alleles display highly penetrant ISNb bypass (79%, n = 58 embryonic stage 16 A2–A7 hemisegments for ena^{210}/ena^{210} and 96%, n = 190 for ena^{23}/ena^{23}), demonstrating a requirement for both domains in the guidance mechanism. Interestingly, the inversion allele ena^{GC8} displays a weak dominant-negative effect on ISNb guidance (7% ISNb bypass in $ena^{GC8}/+$, n = 182).

Although Ena is restricted to axons in the developing nervous system late in embryogenesis, it is expressed broadly prior to germ band retraction (Gertler et al., 1995). To confirm that neuronal Ena function is necessary for ISNb choice point navigation, we expressed wild-type *ena* cDNA under neuronal GAL4 control in an ena^{GC1}/ena^{GC5} mutant background. Neural specific *ena* expression attenuated the ISNb phenotype significantly; ena^{GC1}/ena^{GC5};P[neu-GAL4]/P[UAS-*ena*] embryos displayed 29% bypass (n = 73), compared with 86% in ena^{GC1}/ena^{GC5} alone (see above). If the quantity of Ena protein is rate limiting in wild-type ISNb axons, we might expect Ena overexpression to disrupt ISNb guidance. However, we observed no ISNb phenotypes, even when UAS-*ena* was combined with the strongest neural driver P[elav-GAL4] (data not shown).

Ena Associates Directly with Dlar PTP Domain D2

The genetic relationship between Abl and Dlar and the requirement of Ena function for ISNb target entry suggest that Ena might act in the Dlar signaling pathway. To test this model, we asked whether Ena associates with the cytoplasmic domain of Dlar. We exposed equal amounts of different GST–PTP fusion proteins to *Drosophila* S2 cell lysates and found that endogenous Ena protein associates with a Dlar full-length cytoplasmic domain (GST–Dlar D1–D2) or with D2 alone but not comparably with wild-type D1 (Figure 5A). Since Abl is known to associate with Ena (Gertler et al., 1995), and we had demonstrated binding between Abl and Dlar, it was possible that Ena binding to Dlar required Abl or additional proteins. Therefore, we repeated the GST pull down assays using purified, recombinant Ena protein

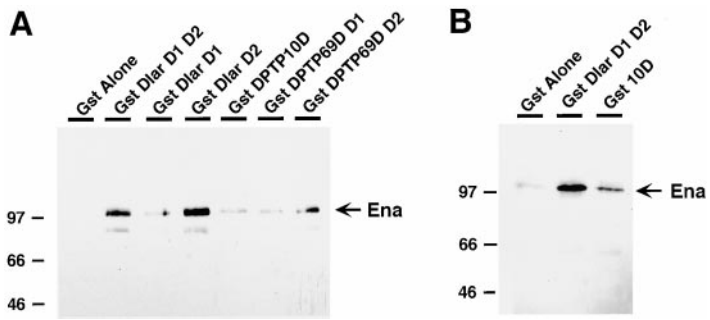

Figure 5. Enabled Protein Associates with the Cytoplasmic Domains of Dlar and DPTP69D

(A) Endogenous full-length Ena protein (arrow) expressed in S2 cell lysates associates with GST fusion proteins that contain the cytoplasmic domain of Dlar and DPTP69D, as visualized by Western blot with anti-Ena antibodies. Equal amounts of each GST fusion protein were loaded in each lane. Lane 1, GST alone; lane 2, GST–Dlar D1–D2; lane 3, GST–Dlar D1; lane 4, GST–Dlar D2; lane 5, GST–DPTP10D; lane 6, GST–DPTP69D D1; and lane 7, GST–DPTP69D D2.

(B) Purified, recombinant Ena (arrow) binds to GST–Dlar D1–D2 (lane 2), and binds weakly to GST–DPTP10D (lane 3) but does not bind significantly to GST alone (lane 1).

Molecular weight markers are shown in kilodaltons.

in the absence of other *Drosophila* proteins. Purified Ena bound to the Dlar cytoplasmic domain (Figure 5B). In both extract and recombinant protein binding assays, Ena showed only weak association with DPTP10D. However, Ena bound effectively to the D2 domain of DPTP69D (Figure 5A). The preferential binding of Ena to the D2 domains of Dlar and DPTP69D, as compared with the D1 domains of the same RPTPs, suggests that these interactions are specific. The parallel between Dlar and DPTP69D binding is interesting, given the published observation that DPTP69D is required for ISNb guidance and can partially substitute for Dlar in vivo (Desai et al., 1997a). Furthermore, the nature and penetrance of ISNb defects in *ena* mutants suggests that Ena may function downstream of multiple inputs.

Abl and Ena Are Substrates for Dlar In Vitro

The relationships between Abl, Ena, and Dlar in motor axon guidance suggest a model whereby Abl and Dlar compete for shared substrates to regulate growth cone behavior. Although the Dlar cytoplasmic domain was previously shown to encode an active PTP domain, using artificial phospho-peptide substrates in vitro (Streuli et al., 1989), no physiological substrates have been identified. Since nearly all of the tyrosine phosphatase activity of LAR family RPTPs resides in the D1 domain, we examined the ability of the GST–Dlar D1 fusion protein to dephosphorylate purified *Drosophila* Abl or Ena proteins after these proteins had been phosphorylated with recombinant d-Abl and ^{32}P–ATP (see Experimental Procedures). Incorporated ^{32}P was rapidly released from both Abl and Ena after addition of wild-type GST–Dlar D1 but not after addition of the catalytically inactive C-to-S mutant GST–Dlar D1 fusion protein (Figure 6). These results suggest that the bacterially expressed GST–Dlar protein is correctly folded and that *Drosophila* Abl and Ena are both potential Dlar substrates. However, because PTPs are known to be promiscuous in vitro, additional experiments will be necessary to ask whether Abl and/or Ena are targets for Dlar activity in vivo.

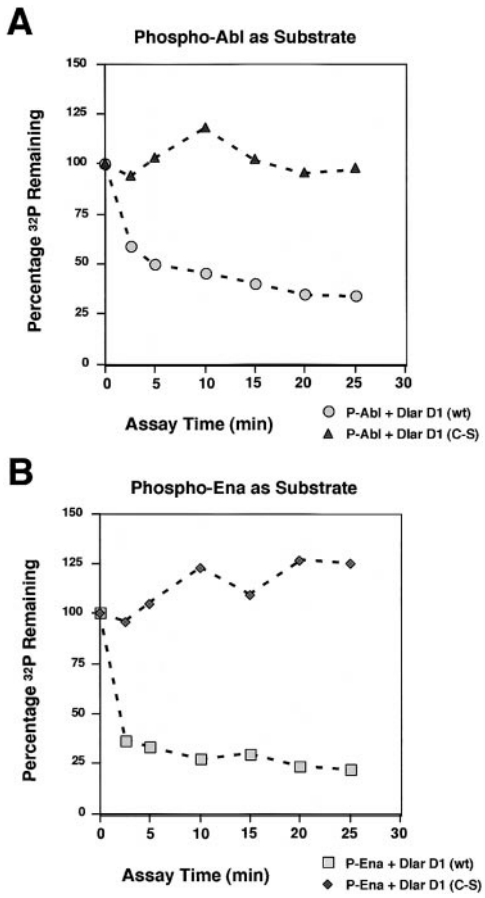

Figure 6. Abl and Ena Are Substrates for the Dlar Phosphatase In Vitro

(A) This assay measures the amount of incorporated ^{32}P remaining over time on a phosphorylated target protein after exposure to Dlar D1 domain fusion proteins (see Experimental Procedures). In this panel, purified, recombinant d-Abl was autophosphorylated prior to the phosphatase assay. A catalytically inactive C-to-S *Dlar* mutant is used as a control (triangles). Phosphate is lost rapidly when d-Abl is mixed with wild-type GST–Dlar D1 (circles).

(B) Purified, recombinant Ena that was phosphorylated by d-Abl in vitro is an excellent substrate for GST–Dlar D1 (wt) in the same assay. The increase in signal seen in the GST–Dlar D1 (C–S) control is likely to reflect an incomplete inhibition of tyrosine kinase activity in this assay (see Experimental Procedures).

Discussion

The ISNb Axon Guidance Pathway

Genetic analysis in *Drosophila* and *C. elegans* has proven to be a powerful tool to identify conserved molecules that control axon guidance decisions (reviewed by Tessier-Lavigne and Goodman, 1996). Several axon guidance choice points have emerged in each organism as key model systems in which genetic and molecular tools can be applied to a well defined navigational problem. ISNb motor axon guidance is one such model. Forward and reverse genetic screens have defined a number of genes required for different aspects of ISNb defasciculation and/or target entry (e.g., Sink et al., 1993, Soc. Neurosci., abstract; Van Vactor et al., 1993; Desai et al., 1996; Fambrough and Goodman, 1996; Krueger et al., 1996; Korey et al., 1997, Soc. Neurosci., abstract; Kaufmann et al., 1998; Winberg et al., 1998; Yu et al., 1998). This approach has been very successful in revealing transmembrane or secreted proteins likely to mediate cell–cell interactions; at least eight such genes have been found to control ISNb guidance. However, little is known about the intracellular signaling machinery used to generate accurate guidance responses. One important clue has come from the fact that three of the ISNb choice point genes contain PTP domains, implicating phosphorylation as an element in the signaling process (reviewed by Desai et al., 1997b; Van Vactor, 1998a; Van Vactor et al., 1998). Although the role of tyrosine phosphorylation is likely to be central for axon guidance in many systems (reviewed by Van Vactor, 1998b), we still understand little about growth cone signaling pathways.

To begin unraveling the Dlar pathway controlling ISNb axon guidance, we have used dose-dependent genetic interactions as a means of finding candidate signaling partners. By searching for a relevant tyrosine kinase in

the pathway, we discovered an antagonistic relationship between Dlar and *Drosophila* Abl. Loss of Abl function suppresses the Dlar axon guidance phenotype, and gain of Abl function mimics this phenotype. The relationship between Dlar and Abl appears to be direct, since purified Abl binds to and phosphorylates the Dlar cytoplasmic domain. Unlike some Abl functions, its role in ISNb development is dependent upon an active tyrosine kinase domain (see Wills et al., 1999). Consistent with these observations, we also find that the Abl substrate protein Enabled is required for ISNb target entry. Like Abl, purified Ena binds directly to the Dlar cytoplasmic domain and serves as a Dlar substrate in vitro, further supporting the model that Abl and Ena participate directly in the Dlar pathway. The strength of the evidence for a connection between Dlar and the Abl pathway is primarily genetic and must be confirmed with future experiments to elucidate biochemical interactions in vivo. However, our current observations are important, not only because they identify proteins that will help us understand the nature of Dlar signaling, but also because they suggest the first direct linkage of Abl and Ena to a specific transmembrane molecule.

Regulation of LAR Family RPTPs

A key unanswered question for the RPTP mechanism concerns the regulation of these molecules by extracellular signals. Despite the size and diversity of the RPTP protein family and the striking conservation in ectodomain structure across species, few ligands have been identified (reviewed by Stoker and Dutta, 1998; Van Vactor, 1998a). Recent work suggests that specific isoforms of vertebrate LAR bind to laminin–nidogen complexes and may mediate cell shape changes (O'Grady et al., 1998). Additional unpublished data suggest that the chicken homolog of LAR, CRYPα, binds to ligands that accumulate in basement membrane surrounding the exit pathway and muscle targets of the spinal motor neurons that express this RPTP (A. Stoker, personal communication). It is interesting that LAR localizes to focal adhesions (FAs), where extracellular matrix (ECM) receptors (e.g., integrins) also concentrate (Serra-Pages et al., 1995). Studies of chick growth cones reveal that FA-like complexes rich in integrins and other proteins (e.g., Paxillin) contain high levels of phosphotyrosine when grown on fibronectin but not on laminin (Gomez et al., 1996). Thus, it is conceivable that PTP activity toward FA-enriched proteins is regulated in some way by laminin binding. Conversely, integrin engagement by fibronectin recruits c-Abl to FAs and activates the kinase within minutes, pushing phosphorylation in the opposite direction (e.g., Lewis et al., 1996). Perhaps then it is no surprise that Mena also localizes to FAs (Gertler et al., 1996), where it may interact with and be regulated by the antagonistic activities of Abl (and Arg) and LAR family RPTPs.

The Role of Ena in the Guidance Pathway

In neuronal growth cones, Mena is discretely localized to filopodial tips, exactly where one might expect it to coordinate the first response to axon guidance cues (Lanier et al., 1999 [this issue of *Neuron*]). The near complete penetrance of ISNb bypass in strong *ena* mutants demonstrates that Ena function in ISNb growth cones is absolutely necessary for correct innervation of target muscles. This contrasts many of the neuronal cell surface components that govern ISNb choice point navigation, such as Dlar, DPTP69D, and DPTP99A (Desai et al., 1996; Krueger et al., 1996), each of which displays only partial requirements. This suggests that Ena acts as a point of convergence downstream of several inputs that collaborate to yield highly accurate guidance behavior. Double mutant analysis and functional substitution experiments suggest that Dlar and DPTP69D share downstream components (Desai et al., 1997a). The parallel in Ena protein association with the D2 PTP domains of Dlar and DPTP69D supports this idea. Whether Ena might also contribute to the function of other proteins, like Semaphorin-I, Plexin A, and/or Beaten-path is unclear at this time.

The association of Abl and Ena with the Dlar cytoplasmic domain and the genetic data implicating them in a common mechanism raise the question of whether Ena is a substrate of Dlar in vivo, as it appears to be in vitro. To date, no physiological substrates have been found for LAR family RPTPs. Abl has been shown to phosphorylate six Ena tyrosines when examined in S2 culture cell lysates (Comer et al., 1998), although this pattern may differ from the pattern observed in neuronal cell types. When all six mapped sites are simultaneously mutated in a P[*ena*$^{(Y\text{-}F6)}$] construct, there is a 40% reduction in transgene-mediated rescue of lethality in *ena* mutant backgrounds (Comer et al., 1998). However, none of these phosphoacceptor sites are conserved in the closest mammalian relative, Mena (Gertler et al., 1996). In contrast, preliminary data from phospho-peptide library chromatography, which seeks an optimal peptide partner for the Dlar D1 domain, reveal a weak consensus that matches an Ena motif completely conserved in the vertebrate proteins Mena, Evl, and VASP (M. Yaffe et al., unpublished data). Extensive biochemical and mutagenesis experiments are necessary to test the functional requirement for this and other potential sites. Of course, although Ena is required for choice point navigation, it may not be the functional target of Abl kinase activity in this context. In this light, it is intriguing that Dlar itself appears to be a good substrate for Abl in vitro.

Axon Guidance through Links to Cytoskeleton

Now that links between the cell surface and the intracellular transducers of guidance information are emerging, one wonders how these signals are conveyed to their ultimate effector systems within the growth cone. The role of Ena in choice point navigation suggests that the actin cytoskeleton is central in this process. Elegant studies of the actin-based motility of Listeria monocytogenes suggest that Ena family members influence cytoskeletal dynamics by promoting actin assembly (reviewed by Pollard, 1995). Furthermore, overexpression of Mena in fibroblasts is sufficient to induce actin-rich protrusions (Gertler et al., 1996). Of course, it has been known for some time that actin is required for the directional specificity of axon outgrowth (Letourneau and

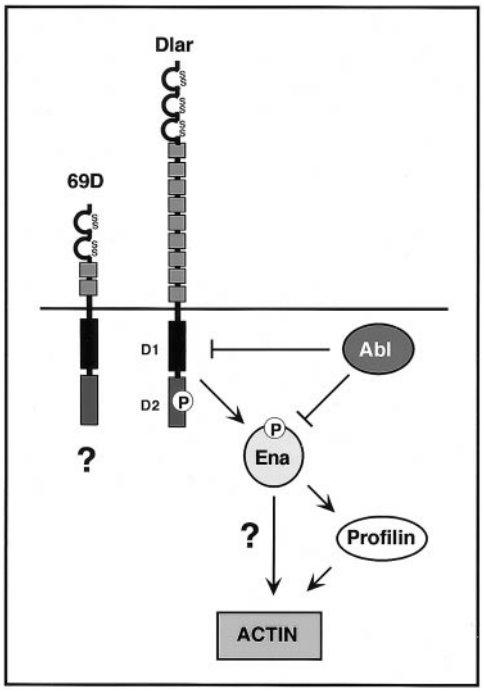

Figure 7. A Genetic Model for the Dlar Signaling Mechanism

Dlar, and also DPTP69D, direct ISNb axons into the correct target domain. Since the loss-of-function phenotypes of *Dlar* and *ena* block target entry, resulting in ISNb bypass, as does a partial blockade of actin assembly with cytochalasin D (Kaufmann et al., 1998), we propose that Dlar and Ena function to promote actin assembly. Abl kinase activity antagonizes the action of Dlar and Ena, presumably by the phosphorylation of these or other proteins. We anticipate that Profilin will also participate in this pathway, given the demonstrated physical associations between Profilins and Ena family members (Gertler et al., 1996), in addition to the dramatic genetic interactions between Abl and Profilin (Wills et al., 1999) and Mena and Profilin (Lanier et al., 1999). Whether Ena has additional partners that mediate cytoskeletal or other functions is unknown.

Marsh, 1984; Bentley and Toroian-Raymond, 1986). Accordingly, partial pharmacological blockade of actin assembly in *Drosophila* embryos results in ISNb bypass phenotypes reminiscent of *ena* and *Dlar* loss of function (Kaufmann et al., 1998). One candidate that may link Ena to actin assembly is Profilin, a small protein that associates with all Ena family members, binds directly to actin, and influences actin assembly (reviewed by Theriot and Mitchison, 1993). In a companion manuscript, we show that mutations in *Drosophila* Profilin (*chickadee*) result in an ISNb growth cone arrest phenotype identical to the ISNb phenotype that we recently discovered in *abl* loss-of-function mutants (Wills et al., 1999). The striking dose-sensitive genetic interaction between *abl* and *chickadee* mutations that we observe in the *Drosophila* nervous system provides suggestive evidence that Profilin is controlled in some way by Abl activity. Interestingly, genetic interactions confirm a functional relationship between Mena and Profilin in the mouse embryo (Lanier et al., 1999).

Conclusions

Our genetic data predict a model whereby Dlar and other choice point genes function to control growth cone behavior by influencing the actin cytoskeleton (Figure 7). Some proteins in the pathway are likely to foster actin assembly, such as Ena and perhaps Dlar, while others may antagonize this process, such as Abl. Consistent with this model, mice lacking both Abl and its close relative Arg display excessive accumulations of microfilaments in the developing nervous system (Koleske et al., 1998). Other cytoskeletal signaling factors are also involved in ISNb guidance, such as the small GTPase Rac1 (Kaufmann et al., 1998). However, it is not yet clear how protein tyrosine phosphorylation will control or coordinate the activities of different intracellular proteins. Certainly, the reversibility of phosphorylation makes it ideal for the integration of cues across a complex and changing landscape and for dynamic processes whereby components such as actin need to recycle.

Experimental Procedures

Genetics and Anatomical Analysis

Gal4 expression was controlled by the neuronal specific P[C155-GAL4], P[1407-GAL4], P[elav-GAL4], or P[neu-GAL4] driver chromosomes (1407 and elav were obtained from Dr. Y. N. Jan, C155 from Dr. C. S. Goodman, and neu from Dr. N. Perrimon). All flies were maintained at 25°C. Motor axon pathways were visualized with mAb 1D4 as previously described (Van Vactor et al., 1993). Motor axon bypass is scored when some or all ISNb axons fail to enter the ventral domain between muscles 15 and 14/28 and extend beyond this entry point; using this criterion, there is a low level of ISNb bypass in wild-type embryos (1%–5%, depending on genetic background). mAb 3C10 (anti-even-skipped) and mAb In 4D9B1F1 (anti-engrailed) were used to assess cell fate and patterning in the CNS (obtained from Dr. Goodman). mAb 2D5 (Patel et al., 1987) was used to asses RP1, RP3, and RP4 development. When homozygous mutants could not be made, the correct genotypes were chosen by staining with anti-*lacZ* antibodies (Sigma) to recognize embryos carrying *lacZ*-expressing balancer chromosomes (CyO-P[actin5C-*lacZ*] and/or TM3-P[actin5C-*lacZ*]). Prior to evaluation, precisely staged embryos of the appropriate genotypes were selected and filleted under a dissection microscope. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Due to the strong, broad staining pattern of mAb 2D5 (anti-Fas III), the analysis of RP axon trajectory was performed without the anti-*lacZ* counter stain for balancer chromosomes. In crosses between P[elav-GAL4]/P[elav-GAL4] and P[UAS-*abl*]/TM6B stocks, half of the embryos will misexpress Abl. Only three examples of RP axon defects were observed in 337 embryonic stage 15–16 hemisegments from P[elav-GAL4];P[UAS-*abl*] crosses; this corresponds to a penetrance of 2%. Twelve examples of abnormal RP axon trajectories were observed in 449 embryonic stage 15–16 hemisegments from a cross between ena^{GC5}/*CyO* and ena^{GC8}/*CyO* in the absence of a counter stain to identify balancer chromosomes; since only 25% of embryos in these collections are ena^{GC5}/ena^{GC8}, this frequency is equivalent to a penetrance of 11%.

Cloning and Expression of Phosphatase Fusion Proteins

The wild-type intracellular PTP domains of Dlar (D1 [amino acids 1452–1766], D2 [amino acids 1738–2030]) and DPTP69D (D1 [amino acids 900–1190], D2 [amino acids 1191–1480]) were amplified by polymerase chain reaction (PCR) and subcloned into the polylinker of pGEX-1 (Pharmacia). GST–DPTP10D (pGEX-2T), representing the entire intracellular domain, was obtained from Dr. Kai Zinn (Tian and Zinn, 1994). Cysteine-to-serine (C–S) mutations to inactivate the PTP catalytic domains were constructed by using a PCR-based mutagenesis protocol (N. Krueger, personal communication), and all PCR-derived constructs were DNA sequenced. Fusion proteins were isolated from bacterial lysates as suggested by the manufacturers of the GST purification module (Pharmacia). Glutathione-eluted proteins were dialyzed and stored at 4°C in 50 mM HEPES (1 mM EDTA/1 mM EGTA/1 mM $MgCl_2$/25 mM KCl/1 mM DTT in

50% glycerol, pH 7.6). Protein concentrations were determined by Bradford-Lowry assays (Biorad) in addition to silver- and Coomassie staining of proteins on SDS–PAGE gels.

Protein Association Assays with S2 Cell Extracts

Schneider cells (S2; American Type Culture Collection) were maintained at 22°C in Schneider's medium (Gibco/BRL) supplemented with 10% fetal calf serum (hat-inactivated) and penicillin/streptomycin (50 μg/ml). In association experiments, 2×10^6 cells per μg of GST protein assayed were lysed in NPTE buffer (1% NP-40/150 mM NaCl/50 mM Tris-Cl, pH 7.8/10 mM DTT/1 mM $MnCl_2$/5 mM EDTA/1 mM Pefabloc [Boeringer Mannheim], plus 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Samples were lysed by a 10 s vortex and placed at 0°C for 25 min, and cell debris was pelleted and removed by a 20 min 13,000 × g spin at 4°C. Lysates were precleared with 1 μg of GST alone incubated at 24°C, with agitation for 20 min. Lysates were then incubated with GST fusion proteins at 24°C for 1 hr prior to pull down with glutathione-Sepharose beads (Pharmacia). GST-bound Sepharose pellets were washed three times with NPTE buffer and were then boiled in sample buffer (Laemmli, 1970) prior to SDS–PAGE (6.5% or 10% polyacrylamide) and subsequent transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore) by wet transfer in 25 mM Tris-Cl/192 mM glycine/10% methanol (pH 8.3). Abl and Ena Western blots were performed as previously described (Gertler et al., 1995). GST Western blots were carried out with anti-GST antibody (Pharmacia) as suggested by the manufacturer. All secondary antibodies (Sigma; HRP-conjugated) were used at a 1:10,000 dilution and visualized by enhanced chemiluminescence (Amersham).

Protein Association Assays with Purified Proteins

Purified recombinant His-tagged Ena and Abl protein was prepared by affinity to Ni-NTA agarose (Qiagen) from baculovirus-infected SF9 cell lysates as described by Comer et al. (1998). In association studies with Ena, 5 nmol of protein was used in combination with 1 μg each of GST fusion proteins and the same protocol used in extract experiments (see above). For v-Abl (New England Biolabs [NEB]) or d-Abl, 1–2 nmol of protein was first autophosphorylated in NEB buffer (vAbl) or kinase buffer (d-Abl) (20 mM PIPES/10 mM $MgCl_2$/10 mM $MnCl_2$/1 mM DTT/10 mM ATP [pH 6.9], both containing 20 μCi (γ^{32}P)-ATP (Amersham). Subsequent associations were carried out as above except in NPT buffer (1% NP-40/150 mM NaCl/50 mM Tris-Cl, pH 7.4/5 mM DTT/1 mM Pefabloc, plus 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Proteins were visualized after SDS–PAGE by autoradiography and subsequent Western blot analysis.

PTP Assay

One hundred nanograms of the designated protein substrate (per PTP reaction) was tyrosine phosphorylated in the presence of γ^{32}P-ATP as described in the protein association assay protocol. Five micrometers of an erbstatin analog (Calbiochem) was then added to the phosphorylated substrate in order to inactivate Abl tyrosine kinase activity (10 min at 30°C). Substrate proteins were then diluted in PTP buffer (10 mM Tris/10 mM EDTA/5 mM DTT [pH 7.4], a t = 0 time point sample was taken, and 1 ug of the GST–Dlar fusion protein (either wild-type or C–S) was added. The PTP reaction was incubated at 30°C, and samples were taken at designated time points. Sample buffer was added to each sample, tubes were boiled for 5 min, and proteins were run out on SDS–PAGE. Gels were subsequently transferred as described earlier. Protein tyrosine phosphorylation (^{32}P incorporated into the target protein) was measured from exposed phosphoimaging plates with Fuji phosphoimaging software. Equal loading of samples was subsequently verified by Western blot analysis.

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