Interactions between the secreted protein Amalgam, its transmembrane receptor Neurotactin and the Abelson tyrosine kinase affect axon pathfinding

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SUMMARY

Two novel dosage-sensitive modifiers of the Abelson tyrosine kinase (Abl) mutant phenotype have been identified. Amalgam (Ama) is a secreted protein that interacts with the transmembrane protein Neurotactin (Nrt) to promote cell:cell adhesion. We have identified an unusual missense ama allele, ama¹⁰⁹, which dominantly enhances the Abl mutant phenotype, affecting axon pathfinding. Heterozygous null alleles of ama do not show this dominant enhancement, but animals homozygous mutant for both ama and Abl show abnormal axon outgrowth. Cell culture experiments demonstrate the Ama¹⁰⁹ mutant protein binds to Nrt, but is defective in mediating Ama/Nrt cell adhesion. Heterozygous null alleles of nrt dominantly enhance the Abl mutant phenotype, also affecting axon pathfinding. Furthermore, we have found that all five mutations originally attributed to disabled are in fact alleles of nrt. These results suggest Ama/Nrt-mediated adhesion may be part of signaling networks involving the Abl tyrosine kinase in the growth cone.

Key words: Neural development, Axon pathfinding, Abelson tyrosine kinase, Abl, amalgam, disabled, neurotactin, Drosophila melanogaster

INTRODUCTION

The cytoplasmic tyrosine kinase Abl is incorporated into multiple signaling networks, including those regulating cell cycle progression, cytoskeletal dynamics and axon outgrowth (Van Etten, 1999). In Drosophila, the Abl kinase is localized to the axons of the central nervous system (CNS) during embryogenesis (Bennett and Hoffmann, 1992; Gertler et al., 1989). Thus, genetic screens for second site mutations that alter the Abl mutant phenotype in Drosophila have been instrumental in identifying many components of the networks regulating axon extension (Hoffmann, 1991; Lanier and Gertler, 2000). In these genetic screens, a variety of different experimental approaches have been taken; both enhancers and suppressors of the Abl mutant phenotype have been identified, and genes that show dosage-sensitive interactions with Abl, as well as genes that alter the Abl mutant phenotype only when homozygous mutant, have been uncovered.

One theme that has emerged from these genetic analyses is that the Abl kinase may influence axon outgrowth by affecting cytoskeletal dynamics. Dosage-sensitive genetic interactions affecting axon outgrowth have been found between Abl and enabled (ena), with ena being a dominant suppressor of the Abl mutant phenotype (Gertler et al., 1990). ena encodes an Abl substrate with multiple SH3-binding sites (Gertler et al., 1995) and was the first identified member of the Ena/VASP family (Lanier and Gertler, 2000). Its mammalian homolog Mena plays roles in axon guidance and cell migration by affecting actin filament assembly (Bear et al., 2000; Bear et al., 2002; Gertler et al., 1996; Goh et al., 2002; Lanier et al., 1999; Renfranz and Beckerle, 2002). Dosage-sensitive genetic interactions affecting axon outgrowth have also been found between Abl and trio, with trio being a dominant enhancer of the Abl mutant phenotype (Liebl et al., 2000). trio encodes a protein with multiple domains, including an SH3 domain and two guanine-nucleotide-exchange-factor domains (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000), which can function to reorganize the actin cytoskeleton (Newsome et al., 2000). Abl and the profilin homolog chickadee show dosage-sensitive genetic interactions affecting axon outgrowth, with chickadee dominantly enhancing a motoneuron phenotype in Abl mutants (Cooley et al., 1992; Wills et al., 1999). A protein with similarities to neurofilaments, fiax axon connections (fiax), has also been identified as a dominant enhancer of the Abl mutant phenotype (Hill et al., 1995).

Abl has recently been shown to have multiple interactions with robo signaling networks. robo encodes the transmembrane receptor for Slit and is involved in regulating

*Accepted 14 April 2003*
axon midline crossing (Kidd et al., 1999; Seeger et al., 1993). Bashaw et al. found that in a background sensitized by overexpression of Abl, robo serves as a dominant enhancer with heterozygous mutations strongly increasing inappropriate midline crossing in the CNS, leading to a model whereby Robo is negatively regulated by Abl phosphorylation (Bashaw et al., 2000). Others have also shown that Abl and Capt (a protein involved in actin cytoskeleton dynamics) may be involved in restricting midline crossing in response to Slt (Willis et al., 2002). Additionally, it has been shown that Abl may phosphorylate β-catenin, negatively regulating N-cadherin function in response to Slt-activated Robo (Rhee et al., 2002).

A dosage-sensitive genetic interaction that affects axon outgrowth between Abl and disabled (dab) has been reported, with dab dominantly enhancing the Abl mutant phenotype (Gertler et al., 1989), dab, which is the ortholog of murine Dab1 (Howell et al., 1997) or scrambler (Ware et al., 1997), encodes a tyrosine phosphorylated adaptor protein containing a phosphotyrosine-binding (PTB) domain (Gertler et al., 1993; Howell et al., 1997). Work in the murine system has shown Dab1 plays a role in neuronal migration (Gallagher et al., 1998; Ware et al., 1997).

This report now expands and modifies the cast of characters included in axon extension networks involving Abl. We show that amalgam (ama) and neurotactin (nrt) interact genetically with Abl. ama encodes a secreted protein with three Ig-domains (Fremion et al., 2000; Seeger et al., 1988), while nrt encodes a transmembrane protein with a catalytically inactive cholinesterase domain (Barthalay et al., 1990; de la Escalera et al., 1990; Hortsch et al., 1990) recently identified as the in vivo receptor for Amalgam (Fremion et al., 2000). We have identified an unusual missense allele of ama generated in genetic screens for strong dominant enhancers of the Abl mutant phenotype. In addition, our molecular characterization of chromosomes carrying dominant enhancers of the Abl mutant phenotype, originally identified as dab alleles, has shown that these chromosomes in fact are mutant for nrt. These mutations all show dosage-sensitive effects on axon pathfinding in the Abl mutant background. As the binding of Amalgam to Neurotactin promotes cell adhesion (Fremion et al., 2000), these genetic interactions define an additional role for the Abl tyrosine kinase in axon guidance.

MATERIALS AND METHODS

Genetics

All genetic crosses were carried out using standard cornmeal-yeast medium in humidified, 25°C incubators. The ama M109 allele was recovered in genetic screens which mutated the Abl1 chromosome with ethylmethane sulfonate (EMS) as previously described (Gertler et al., 1989; Hill et al., 1995). For all dosage-sensitive genetic tests, stocks were balanced over the TM6, B, Hu, e, Tb, ca balancer chromosome. The percent of expected Th+ pupae recovered from each cross was calculated by dividing the total number of Th+ pupae by one half the total number of Th+ pupae. The percent of expected Hu+ adults recovered from each cross was calculated in the same way. All data reported represent a minimum of 400 progeny counted from each cross.

Sequence analysis

In all cases genomic DNA was isolated from pupae or animals hemizygous for the gene of interest. All amplifications for sequence analysis were carried out using polymerase with proofreading activity (Advantage HF2, BD Biosciences/Clontech, Palo Alto, CA). The ama ORF was amplified as one 1.3 kb piece. The nrt ORF was amplified as four overlapping fragments, each ~800 bp. The dab ORF was amplified as eleven fragments, each ~850 bp. Not all of these fragments overlapped, as not all of the two large introns (intron 1 and intron 4) of dab were sequenced. Sequences of primers used are available on request. All fragments showing a deviation from wild type were independently re-amplified and re-sequenced.

RNAs and immunostaining

dsRNA was generated and injected into embryos as described (Kennerdell and Carthew, 1998), with the exception that embryos were injected dorsally. Primer sequences used to generate templates for in vitro transcription of Abl, nrt and ama are available upon request. After injection, embryos were raised at 18°C, and harvested at stage 14-15 for mAb BP102 immunohistochemistry (Patel et al., 1987). mAbs BP102 and BP106 were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-β-galactosidase mAb (Promega, Madison WI) was used to detect lacZ expression from enhancer-trap-containing balancer chromosomes to distinguish the genotypes of the embryos.

S2 cell assays

All ama and nrt alleles were cloned into the pMET vector (Bunch et al., 1998), under the control of the metallothionine promoter. Stable S2 cell populations were derived by co-transfecting with pPC4 (Elkins et al., 1990) and selecting for α-amanitin resistance. Fragments containing the mutations associated with amaM109, nrt M100 and nrtM221 were introduced into pMET-Ama, pMET-Ama-TM (Fremion et al., 2000) and pMET-Nrt constructs using available restriction enzyme sites and confirmed by DNA sequencing. For aggregation assays, conditioned media was generated by inducing expression of pMET-Ama, pMET-AmaM109 or naïve S2 cells with 0.7 mM CuSO4 overnight. Ama-expressing cells were then removed by centrifugation. The conditioned media was added to naïve S2 cells or cells transfected with different pMET-Nrt constructs. The addition of conditioned media containing 0.7 mM CuSO4 initiated expression of pMET-Nrt. Aggregation assays were conducted in six-well microtiter plates on a rotary shaker at 80 rpm with ~5×106 cells per ml. Particles (cells and aggregates) were counted on a hemacytometer at t=0, 4 and 8 hours. Cells transfected with pMET-Ama-TM and pMET-AmaM109-TM were induced directly with 0.7 mM CuSO4 at the beginning of the aggregation assay. Accumulation of these membrane-anchored forms of Ama was detected with mAb BP104, which recognizes an epitope in the cytoplasmic domain of Nrg (Hortsch et al., 1995).

For cell pull-down assays, expression of Nrt was induced with overnight exposure to 0.7 mM CuSO4. Nrt-expressing cells (~5×106 per ml) were recovered by low-speed centrifugation and resuspended in Ama-conditioned media (see above). Cells were allowed to aggregate for 60 minutes and then recovered by centrifugation. Cell pellets were washed once with Schneider’s media, re-pelleted and re-suspended in Laemmli lysis buffer. Equivalent numbers of cells were used in all assays. Equal volumes of cell lysates were loaded for immunoblot analysis. Nrt was detected with mAb BP106 (Hortsch et al., 1990). Ama was detected with rabbit anti-Ama antisera (Seeger et al., 1988).

RESULTS

An allele of amalgam was isolated as a novel dominant enhancer of the Abl mutant phenotype

Similar to the genetic screens that yielded alleles of fax and trio, third chromosomes carrying the Abl1 allele were mutagenized with EMS, and dominant mutations that shifted
Table 1. Dosage-sensitive modification of the Abl mutant phenotype by ama mutations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dose of Abl</th>
<th>Dose of ama</th>
<th>Pupae*</th>
<th>Adults*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abl1/Abl4</td>
<td>0</td>
<td>2</td>
<td>96</td>
<td>56</td>
</tr>
<tr>
<td>Abl1, amaM109/Abl4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F[Abl1]; Abl1, amaM109/Abl4</td>
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<td>1</td>
<td>103</td>
<td>116</td>
</tr>
<tr>
<td>Abl1, Df(3R)ama/Abl4</td>
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<td>1</td>
<td>92</td>
<td>22</td>
</tr>
<tr>
<td>Abl1, amaR1/Abl4</td>
<td>0</td>
<td>1</td>
<td>104</td>
<td>67</td>
</tr>
</tbody>
</table>

*The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 400 animals was counted in each cross.

the lethality associated with the Abl mutant phenotype to pre-pupal stages were recovered (Gertler et al., 1989; Hill et al., 1995). A mutation, originally designated as M109, was isolated. Meiotic mapping of M109 placed this mutation between st and th, at ~47cM. Complementation analysis across this region showed that M109 failed to complement Df[3R]MAP11, a well characterized deficiency that removes zen, bcd, ama and Dfd (Diederich et al., 1989). Abl1, M109/Df[3R]MAP11 embryos developed normal cuticles (data not shown), making it unlikely that M109 was an allele of zen or Dfd. Based upon the genetic interaction between Abl and M109, and the similar spatial and temporal expression patterns of Abl and ama (Bennett and Hoffmann, 1992; Seeger et al., 1988), we hypothesized that M109 may be an ama allele. We tested this directly by amplifying the ama open reading frame (ORF) from Abl1, M109/Df[3R]MAP11 ‘escaper’ animals, and comparing it to the ama ORF amplified from the parental Abl1 chromosome. A single alteration was detected from the Abl1, M109 chromosome; residue 46 was changed from cysteine to tyrosine (Fig. 2A). As a disulfide bond between C46 and C117 is believed to stabilize the first Ig domain of Ama (Seeger et al., 1988), the C46Y change probably results in a dramatically altered protein product. Having colocalized a missense mutation within a critical bond between C46 and C117 is believed to stabilize the first Ig domain of Ama (Seeger et al., 1992), we hypothesized that Ama might be a dominant enhancer of the Abl mutant phenotype. However, in situ hybridization to the relevant genes showed that mutations in ama did not enhance the Abl mutant phenotype.

A null allele of ama did not enhance the Abl mutant phenotype

To date, the only ama allele recovered as a dominant enhancer of the Abl mutant phenotype is amaM109. To test whether a null allele of ama could function as a dominant enhancer of the Abl mutant phenotype, we generated a recombinant Abl1, Df(3R)ama chromosome. Df(3R)ama is a small deletion that removes zen, bcd and ama (Fremion et al., 2000). Neither survival to pupation, nor the percentage of segments with commissure defects was dramatically altered between Abl mutant animals and Abl mutant animals heterozygous null for ama. Animals of the genotype Abl1/Abl4 produced 96% of the expected pupae (Table 1), and 3% of the segments in such embryos showed commissure defects (n=240; Fig. 1A). Animals of the genotype Abl1, Df(3R)ama/Abl4 produced 92% of the expected pupae (Table 1), and 2% of the segments in such embryos showed commissure defects (n=222; Fig. 1C). We therefore concluded that a null allele of ama was not a dominant enhancer of the Abl mutant phenotype.

Null point-alleles of ama have never been described. In order to generate such alleles, we capitalized on our observation that a null allele of ama was not a dominant enhancer of the Abl mutant phenotype, and re-mutagenized the Abl1, amaM109 chromosome with EMS, screening for revertants of the...
dominant enhancement by \(ama^{M109}\) of the \(Abl\) mutant phenotype. Ten EMS-induced intragenic revertants of \(ama^{M109}\), all carrying the C46Y \(M109\) mutation in addition to new alterations, have been recovered in this screen. Interestingly, nine of these revertants carry single missense mutations. We are currently characterizing the proteins encoded by these altered \(ama\) genes. However, one allele of \(ama\), an R103C missense allele combined with a 29 bp deletion beginning in the P114 codon, was also generated. This is a null allele, as it can code for only the first 113 residues of Ama (with C46Y and R103C) followed by 54 novel residues due to the frameshift of the 29 bp deletion. We have named this revertant allele \(ama^{R1}\) (Fig. 2A).

We have found that 4% of the segments of embryos of the genotype \(Abl^1, ama^{R1}/Abl^4\) have defective commissures (\(n=176\); Fig. 1D), and all of these embryos survive to pupation (Table 1). In both cases, these data were very similar to the characteristics of the \(Abl^1, Df(3R)ama\) chromosome (2% defective segments, 92% survival to pupation) and very different from the parental \(Abl^1, ama^{M109}\) chromosome (31% defective segments, 0% survival to pupation). We have observed better survival to adulthood of \(Abl^1, ama^{R1}/Abl^4\) animals as compared to \(Abl^1, Df(3R)ama/Abl^4\) animals (Table 1), but as the \(Abl^1, Df(3R)ama\) chromosome was generated by recombination and the \(Df(3R)ama\) deficiency is a multigenic deletion, this discrepancy may be attributable to differences in genetic backgrounds, and we have not investigated this observation further. The recovery of a null allele of \(ama\) in a screen for revertants of the dominant enhancement by \(ama^{M109}\) of the \(Abl\) mutant phenotype confirms our identification of the \(ama^{M109}\) allele as the causative mutation responsible for the dominant enhancement of the \(Abl\) mutant phenotype.

Biochemical and genetic characterization of the \(ama^{M109}\) allele

Fremion et al. (Fremion et al., 2000) have demonstrated that Ama binding to Nrt mediates cell:cell adhesion. These authors have also shown that Ama proteins can self-associate, suggesting a model whereby the binding of Ama to cell-surface Nrt, as well as Ama:Ama homophilic binding somehow cooperate to promote cell:cell adhesion. Similar to Fremion et al., we have employed two assays to examine the biochemical behavior of Ama proteins. First, using S2 cells engineered to express wild-type Nrt (see Materials and Methods), we have tested whether media

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**Fig. 2.** Biochemical characterization of Ama+ and Ama^{M109}.

(A) The organization of Ama with its signal sequence and three Ig domains (loops). The N terminus is towards the left. The \(ama^{M109}\) and the \(ama^{R1}\) mutations are also indicated.

(B) Representative photomicrographs showing our S2 cell adhesion assay at \(t=8\) hours. In the top three panels, all cells were engineered to express wild-type Nrt and were exposed to either control (left), Ama+-containing (center) or Ama M109 -containing (right) conditioned media. In the bottom two panels, cells were engineered to express membrane-anchored forms of Ama.

(C) Quantification of our S2 cell adhesion assays. Bars represent the percent of total particles at \(t=0\) hours counted at \(t=8\) hours. Values represent the average of two independent experiments; error bars show the standard error of the mean. (D) Anti-Ama immunoblots. The arrow indicates the mobility of full-length Ama. Lanes 1, 2: equal amounts of Ama+-conditioned media or of Ama^{M109},conditioned media were resolved. These lanes show that comparable amounts of Ama were present in both types of conditioned media. Lanes 3-6: immunoblots of S2 cell lysates from cell pull-down assays. Equivalent amounts of cell lysates were loaded in each lane. Lane 3: naïve S2 cells exposed to Ama+-conditioned media. Lane 4: Nrt-expressing S2 cells exposed to Ama+-conditioned media. Lane 5: naïve S2 cells exposed to Ama^{M109},conditioned media. Lane 6: Nrt-expressing S2 cells exposed to Ama^{M109},conditioned media. These lanes show that Ama+ and Ama^{M109} bound specifically to S2 cells expressing Nrt. (E) Anti-Nrg immunoblots. Equivalent amounts of cell lysates were loaded in each lane. The arrow indicates the mobility of the Ama-Nrg fusion protein. Lane 1: S2 cells expressing Ama+-TM. Lane 2: S2 cells expressing Ama^{M109}, TM. These lanes show equivalent amounts of these chimeric proteins were expressed.
containing secreted Ama can mediate cell:cell adhesion. Cell adhesion was quantified in these assays by counting total particles over time, with particles defined as single cells and cell aggregates regardless of size. A reduction in particle number indicated cell:cell adhesion, as free cells aggregated into clusters. As part of this assay, we also indirectly monitored Ama:Nrt binding by isolating cells exposed to Ama-containing media, and testing for the specific binding of Ama to Nrt-expressing cells in a cell pull-down assay. For our second assay, we generated Ama proteins fused to the transmembrane and cytoplasmic domains of Neuroglian, thus creating a membrane-anchored Ama (Fremion et al., 2000). By inducing the expression of these chimeric proteins, we tested whether Ama:Ama interaction occurred by monitoring cell aggregation. Both Ama⁺ and AmaM109 were tested in these assays.

While naïve S2 cells did not express Ama, after transfection with the appropriate expression plasmid both Ama⁺ and AmaM109 were produced as stable, secreted proteins at comparable levels (Fig. 2D, lanes 1, 2). Naïve S2 cells did not express Nrt (Fig. 4C, lane 1). Therefore, neither Nrt-expressing S2 cells exposed to naïve supernatant, nor naïve S2 cells exposed to Ama⁺ or AmaM109-containing supernatant showed aggregation (Fig. 2C), and naïve S2 cells did not bind Ama (Fig. 2D, lanes 3, 5). Ama⁺ did bind to Nrt-expressing S2 cells (Fig. 2D, lane 4), and supported their aggregation, with large aggregates of cells forming (Fig. 2B); total particle number was reduced by 48% over the 8 hour assay (Fig. 2C). AmaM109 also bound Nrt-expressing S2 cells (Fig. 2D, lane 6), but the aggregation it induced was clearly different from that seen with Ama⁺ with the aggregates that formed being small (Fig. 2B). In this case, total particle number was reduced by only 23% over the 8 hour assay (Fig. 2C). In addition, Ama⁺-induced aggregates were less stable, disappearing after 24 hours, while AmaM109-induced aggregates persisted beyond 24 hours (data not shown).

Fig. 2 also shows our tests of the ability of Ama⁺ and AmaM109 to self-aggregate when expressed as transmembrane chimeras. Both were expressed at comparable levels (Fig. 2E, lanes 1, 2). Consistent with its ability to support Nrt-mediated cell adhesion as a secreted protein, Ama⁺-TM demonstrated homophilic association (Fig. 2B), with total particle number reduced by 34% over the 8 hour assay (Fig. 2C). However, AmaM109-TM did not induce cell aggregation in this assay (Fig. 2B,C). Taken together, these results suggest that the first Ig-domain of Ama, which is stabilized by the disulfide bond between C46 and C117, is crucial for Ama:Ama interaction, but not for Ama:Nrt interaction. These results also support a model in which Ama:Ama interaction is important for normal Ama:Nrt-mediated cell adhesion (Fremion et al., 2000).

Given the ability of AmaM109 to bind Nrt but mediate only limited cell:cell adhesion, and our observations that the amaM109 allele behaved as a dominant enhancer of the Abl mutant phenotype, while null alleles of ama did not, we wished to explore the genetic properties of the amaM109 mutation. The dominant activity of AmaM109 could be the result of dominant-negative effects. In this model, AmaM109 binding to Nrt exerts its influence by the exclusion of Ama⁺ binding, precluding normal Ama⁺-mediated cell:cell adhesion. Alternatively, the AmaM109 protein could have a more complex biochemical activity, actively producing phenotypic effects by occupying Nrt receptors. Using our amaR1 allele, we hypothesized that if amaM109 behaved strictly as a dominant-negative, the amaM109/Df(3R)ama and the amaM109/Df(3R)ama phenotypes would be similar. We found, however, a dramatic difference between the amaM109/Df(3R)ama and the amaM109/Df(3R)ama phenotypes in an Abl mutant background. Animals of the genotype Abl¹, amaM109/Ab¹, Df(3R)ama were found to have 23% of segments with defective commissures (n=255; Fig. 1E), while animals of the genotype Abl¹, amaM109/Ab¹, Df(3R)ama had 86% of segments with defective commissures (n=630; Fig. 1F). We therefore concluded that AmaM109 exerts phenotypic effects even in the absence of Ama⁺.

Elimination of Nrt strongly enhances the Abl mutant phenotype

Ama is a secreted protein, while Abi is a cytoplasmic tyrosine kinase. The transmembrane protein Nrt has been shown to be an in vivo receptor for Ama (Fremion et al., 2000). Given the strong genetic interaction between amaM109 and Abi, and the unusual genetic character of amaM109, we were curious to test for genetic interactions between nrt and Abl. Although mutant alleles of nrt exist (Speicher et al., 1998), nrt lies just proximal to Abl on chromosome three, precluding the simple generation of an Abl-mutant, nrt-mutant chromosome by recombination. We therefore elected to use RNAi to generate Abl-null, Nrt-null embryos.

Embryos were injected with double-stranded RNA, fixed and mAb BP102 was used to visualize axon tracts in the CNS. As shown in Table 2 and Fig. 3, elimination of Abl, Ama or Nrt singly did not have a strong effect on the number of segments with commissure defects. Although in buffer-injected control embryos, 3% of their segments displayed commissure defects (n=833; Table 2), Abl-null embryos generated with RNAi showed commissure defects in 6% of their segments (n=645; Table 2; Fig. 3A). Ama-null embryos were found to have 3% of their segments with commissure defects (n=159; Table 2). In accordance with previous data (Speicher et al., 1998), Nrt-null embryos had mild CNS defects, with 8% of their segments having commissure defects (n=383; Table 2; Fig. 3B). Eliminating Abl and Ama together had a synergistic effect, with 32% of segments showing commissure defects (n=350; Table 2). Eliminating Abl and Nrt together had an even more dramatic effect, with 85% of segments showing commissure defects (n=443; Table 2; Fig. 3C). There was also a qualitative difference in the defects found, with the disruptions of the axon scaffold resulting from the elimination of Abl and Nrt being much more severe than with any other combination. Only in this background were breaks in

### Table 2. Phenotypic effects of RNAi for Abl, ama and nrt

<table>
<thead>
<tr>
<th>Double-stranded RNA injected</th>
<th>% defects</th>
<th>n†</th>
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<tbody>
<tr>
<td>Buffer control</td>
<td>3</td>
<td>833</td>
</tr>
<tr>
<td>Abl</td>
<td>6</td>
<td>645</td>
</tr>
<tr>
<td>ama</td>
<td>3</td>
<td>159</td>
</tr>
<tr>
<td>nrt</td>
<td>8</td>
<td>383</td>
</tr>
<tr>
<td>Abl + ama</td>
<td>32</td>
<td>350</td>
</tr>
<tr>
<td>Abl + nrt</td>
<td>85</td>
<td>443</td>
</tr>
</tbody>
</table>

*The percent of the total segments that were judged to have defective commissures is shown. Commissures were defined as defective if more than 75% of commissural axons were absent, or clear pathfinding errors were present.

†Total number of segments scored.
Abl and associated with each genotype are shown. (A) dissected. Representative examples of the overall phenotype generated by RNAi (A–C) or by zygotic genotypes (D–F). Embryos scored as having defective commissures.

Fig. 3. CNS phenotypes of Abl and nrt mutants. Mutants were either generated by RNAi (A–C) or by zygotic genotypes (D–F). Embryos from stage 14/15 were stained with mAb BP106, and CNSs were dissected. Representative examples of the overall phenotype associated with each genotype are shown. (A) Abl null, (B) nrt null, (C) Abl and nrt null, (D) Abl, nrtM54/Abtl, (E) Abl, nrtM100/Abtl, (F) Df(3L)st-j7, nrtM2/Abtl, nrtM54. Percentages below each panel indicate the percent of segments with commissure defects; n=total segments scored. Arrows in D,E show typical examples of segments scored as having defective commissures.

Table 3. Dosage-sensitive modification of the Abl mutant phenotype by nrt mutations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dose of Abl</th>
<th>Dose of nrt</th>
<th>Pupae*</th>
<th>Adults†</th>
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<tr>
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<td>57</td>
</tr>
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<td>88</td>
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<tr>
<td>Abl1, nrtM100/Abtl</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Df(3L)st-j7, nrtM2/Abtl</td>
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<td>15</td>
<td>9</td>
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<tr>
<td>Df(3L)st-j7, nrtM2/Abtl</td>
<td>0</td>
<td>1</td>
<td>40</td>
<td>16</td>
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</tbody>
</table>

*As described in the Results, mutations previously attributed to dab are in fact nrt alleles.
†The percent of the expected progeny class of both pupae and viable adults of the indicated genotype is shown. A minimum of 400 animals was counted in each cross.

Identification of nrt as a dominant enhancer of the Abl mutant phenotype

As our RNAi experiments had shown a clear and strong synergistic effect on axon pathfinding when both Abl and Nrt were eliminated, we hypothesized that nrt may function as a dominant enhancer of the Abl mutant phenotype, similar to fax, trio and amaM106. To test this hypothesis, we needed to generate an Abl-mutant, nrt-mutant chromosome. To achieve this, we initiated a genetic screen similar to that detailed by Speicher et al. (Speicher et al., 1998) in which the Abl1 chromosome was mutagenized with EMS so that chromosomes that no longer produced Nrt, as judged by reactivity with the anti-Nrt mAb BP106 (Hortsch et al., 1990), would be identified. We also noted, however, that nrt lies only ~55kb proximal to dab in polytene band 73C. To test the idea that perhaps some mutations originally attributed to dab could, in fact, be nrt alleles, we also screened all existing dab mutant chromosomes [Df(3L)st-j7, dabM2; Df(3L)st-j7, dabM29; Abl1, dabM54; Abl1, dabM100; Abl1, dabM221] for their ability to produce Nrt, as judged by mAb BP106 reactivity in embryos. Before undertaking this analysis, we confirmed previous reports (Gertler et al., 1989) that these chromosomes showed dominant enhancement of the Abl mutant phenotype, and that this dominant effect was dependent on Abl mutations (Table 3). In addition, we confirmed previous reports (Gertler et al., 1989) that this enhancement of the Abl mutant phenotype was accompanied by axon pathfinding defects in the CNS (Fig. 3D,E).

Staining with mAb BP106 showed that embryos homozygous for Df(3L)st-j7, dabM2, Df(3L)st-j7, dabM29 or the Abl1, dabM54 chromosome failed to produce Nrt, identical to In(3L)std11 control chromosomes. In(3L)std11 removes Abl, dab and nrt (Belote et al., 1990; de la Escalera et al., 1990). By contrast, the Abl1, dabM100, the Abl1, dabM221, and the Abl1 and Df(3L)st-j7 parental chromosomes produced Nrt (data not shown). As these preliminary results suggested that some chromosomes carrying dominant enhancers of the Abl mutant phenotype were Nrt-protein-nulls, we abandoned our genetic screen and carried out careful molecular analyses of these chromosomes.

To explore the possibility further that some mutations attributed to dab could in fact be nrt alleles, we generated animals hemizygous for the dab and nrt alleles carried on these mutagenized chromosomes and sequenced both of these genes. For all five dab mutant chromosomes we recovered ‘escaper’ animals over In(3L)std11 by supplying a single wild-type Abl transposon on the second chromosome. From the genomic DNA of these animals, we amplified and sequenced all exons and intron/exon boundaries of both dab and nrt.

For our sequence analysis of dab, we took the conceptual translation of the dab allele found under the Accession Number NM079395 (GenBank) as our wild-type benchmark. This would result in a Dab protein of 2224 residues. We found numerous polymorphisms in the dab alleles we sequenced compared with this wild-type benchmark. Some of these polymorphisms were common to all five alleles (N620T, V1061M, Q543L, A557T, Y993K). However, in no case did we find a unique mutation that would suggest a mutant dab allele had been generated on any of these chromosomes carrying dominant enhancers of the Abl mutant phenotype.

For our sequence analysis of nrt, we took the conceptual translation of the nrt allele reported by Hortsch et al. (Hortsch et al., 1990) as our wild-type benchmark (GenBank Accession
expressing wild-type Nrt bound Ama. S2 cells engineered to express Nrt M221. Naïve S2 cells did not express Nrt+. Lane 3: S2 cells engineered to express Nrt M100. Lane 4: full-length Nrt. Lane 1: naïve S2 cells. Lane 2: S2 cells engineered to express Nrt, and all Nrt proteins were expressed from the pMET plasmid at comparable levels. (Bottom) Anti-Ama immunoblots. Neither mutant Nrt protein mediated Ama + binding (Fig. 4C, lanes 3, 4) and neither mutant Nrt protein mediated Ama-containing supernatant. Neither mutant Nrt protein mediated Ama binding (Fig. 4B). As different nrt null alleles had been created in different Abl mutant backgrounds, we were able to test the CNS phenotype of homozygous Abl mutant, nrt mutant embryos. As shown in Fig. 3F, embryos of the genotype Df(3L)st-j7, dab M29; Abl1, dab M221 showed disruption of the axon architecture, with 63% of segments showing clear commissure defects (n=187). This phenotype is consistent with, but somewhat less severe than the phenotype generated by RNAi elimination of Abl and Nrt (Fig. 3C).

Taken together, our molecular characterization of both dab and nrt from these chromosomes carrying dominant enhancers of the Abl mutant phenotype strongly suggested the causative mutations in all cases were in nrt and not dab. No mutations in dab were found, while three independent null alleles of nrt and two independent missense alleles that eliminate Nrt function were identified. In light of these molecular data, we propose that these dominant enhancers of the Abl mutant phenotype, previously identified as dab M2, dab M29, dab M54, dab M100 and dab M221 be renamed nrt M2, nrt M29, nrt M54, nrt M100 and nrt M221.

**DISCUSSION**

Genetic screens for second-site modifiers are useful tools for identifying components of signaling networks (Hoffmann, 2002).
1991; Thomas, 1993). Over the past decade, work in Drosophila has identified multiple modifiers of the Abl mutant phenotype. With the exception of the transcription factor prospero (Gertler et al., 1993), all of the dominant modifiers identified have been cytoplasmic and co-expressed with Abl in axons. The biochemical characterization of some of the proteins encoded by these dominant enhancers has lead to an emerging model whereby the Abl tyrosine kinase supplies multiple inputs into actin cytoskeleton dynamics in the growth cone (Lanier and Gertler, 2000).

In this study we report two novel dominant enhancers of the Abl mutant phenotype: ama and nrt. The dosage-sensitive genetic interactions between these genes and Abl provide unique information regarding Abl signaling networks. We have identified five independent nrt alleles that remove Nrt function. Three are null alleles (nrtM2, nrtM29, nrtM54), while two (nrtM100 and nrtM221) are missense alleles that behave as protein nulls (Fig. 4B,C). Thus, simply reducing wild-type Nrt activity in an Abl-null background impairs viability (Table 3), suggesting Abl and Nrt lie within one or more common signaling networks. The fact that these genetic combinations have clear effects on axon pathfinding (Fig. 3D,E), strongly suggests that at least one of these common signaling networks has its in vivo output in the growth cone. This is confirmed by the severe axon guidance phenotype produced by disruption of Abl and Nrt function through RNAi or homozygous zygotic mutation (Fig. 3C,F). Disruption of Abl and Nrt by zygotic mutation resulted in strong, but less severe CNS phenotypes than RNAi, probably as a result of elimination of maternally loaded Abl mRNA (Bennett and Hoffmann, 1992).

It had been shown previously that Ama and Nrt functionally interact to mediate cell:cell adhesion (Fremion et al., 2000). Heterozygous null alleles of ama have no detectable dominant effects on axon pathfinding in an Abl-mutant background (Fig. 1C,D), presumably because the biochemical activity of secreted Ama is not directly associated with the cytoplasmic tyrosine kinase activity of Abl. However, disruption of Abl and Ama by homozygous zygotic mutation (Fig. 1F) or by RNAi techniques (Table 2) did show clear synergistic disruptions of the CNS architecture. As with Abl and Nrt, the RNAi-induced phenotype was the more severe of the two, presumably because of the elimination of maternally supplied Abl mRNA.

The identification of the unusual missense ama allele amaM109 as a strong dominant enhancer of the Abl mutant phenotype, affecting both viability and axon pathfinding (Table 1, Fig. 1B) strengthens our conclusion that Ama, Nrt and Abl are functionally intertwined in the growth cone. AmaM109, which alters a cysteine residue needed to stabilize the first Ig domain of Ama (Fig. 2A), eliminates Ama homophilic adhesion but not the ability of AmaM109 to bind Nrt (Fig. 2B-D), and this is probably responsible for its unique character. The biochemical activity of this protein is clearly not wild type, as its ability to support aggregation of Nrt-expressing S2 cells is impaired (Fig. 2B,C). Interestingly, Zhang and Filbin (Zhang and Filbin, 1998) have previously reported that destabilization of the Ig-domain of myelin-specific protein Po results in a dominant-negative effect. Po is a transmembrane protein with a single, extracellular Ig domain stabilized by a disulfide bond between C21 and C98. Chinese hamster ovary cells engineered to express Po showed strong homophilic adhesion. Co-expression of a mutant Po, with an engineered C21A mutation, disrupted the aggregation mediated by wild-type Po.

Genetically, the amaM109 allele phenocopies heterozygosity for nrt in the Abl/+/Abl+ mutant background. Both genotypes result in 100% pre-pupal lethality (Table 1, Table 3), and both result in approximately one-third of embryo segments having defective commissures (Fig. 1B, Fig. 3D,E). Thus, it seems likely that, whatever its biochemical mode of action, the AmaM109 protein disables Nrt activity in a way that simply reducing the dose of wild-type Ama (by heterozygous null mutation) does not.

To understand the function of Nrt in the CNS better, Speicher et al. (Speicher et al., 1998) carried out an extensive genetic analysis, looking for cell adhesion molecules (CAMs) that are functionally redundant to Nrt. This was achieved by generating animals null for nrt and null for a variety of other CAM-encoding genes in pair-wise combinations. Removal of Nrt does not result in a strong CNS phenotype (Table 2, Fig. 3B) (Speicher et al., 1998). Three different genetic combinations showed synergistic interactions in the CNS: nrt and neuroglian (nrg), nrt and derailed (drl), and nrt and kekkon1 (kek1), with the nrt, nrg combination showing the most profound synergy (Speicher et al., 1998). This work suggests the role of Nrt in CNS cell adhesion is at least partially redundant to Nrg, Drl and Kek1. Interestingly, Elkins et al. (Elkins et al., 1990) report that nrg and Abl have no genetic interaction when the morphology of the CNS is assayed by mAb BP102 staining.

Whether Nrt-mediated adhesion provides novel inputs into Abl-mediated signaling networks in the growth cone or whether Nrt-mediated adhesion represents a novel output of the role of Abl in cytoskeleton dynamics can not be determined by the genetic experiments we have carried out. Intriguingly, Fremion et al. (Fremion et al., 2000) report that deletion of the cytoplasmic region of Nrt eliminates its ability to promote cell:cell adhesion. As many transmembrane cell adhesion molecules require functional interactions with the actin-based cytoskeleton (Petit and Thiery, 2000), it is plausible that Ama:Nrt-mediated adhesion requires interaction of the cytoplasmic region of Nrt with actin-based cytoskeleton components. We are currently conducting molecular genetic screens to identify protein:protein interactions involving the cytoplasmic domain of Nrt to clarify this issue.

Our molecular and genetic characterization of nrt as a dominant enhancer of the Abl mutant phenotype has shown that all five mutations previously attributed to dab are nrt alleles (Fig. 4A). How were these mutations initially attributed to dab? Originally it was observed that deletions that remove Abl as well as genes both proximal and distal to Abl (Df(3L)st100.62, Df(3L)st4, Df(3L)st-e5) or only genes proximal to Abl (In(3L)std11), showed a dominant enhancement of the Abl mutant phenotype (Fig. 5) (Henkemeyer et al., 1987). The EMS-induced mutations M2 and M29 mapped tightly to Df(3L)st-j7 and failed to complement In(3L)std11, leading to the hypothesis that these were alleles of a dominant enhancer gene lying proximal to Abl (Gertler et al., 1989). The key observation that lead to dab seems to have been that Df(3L)stE34 did not exhibit dominant enhancement of the Abl mutant phenotype, while Df(3L)st100.62 did show dominant enhancement of the Abl mutant phenotype (Fig. 5) (Gertler
sequence data confirms the $M_2$, $M_{29}$, $M_{54}$, $M_{100}$ and $M_{221}$ mutations are $nrt$ alleles. These findings will necessitate a re-evaluation of other studies that have reported genetic interactions with these alleles (Giniger, 1998; Hill et al., 1995; Le and Simion, 1998).

Our re-examination of the work of others in light of the fact that these alleles are $nrt$ mutations has revealed a startling fact: there may be yet another dominant enhancer of the Abl mutant phenotype in the 73A-C region. As with $Df(3L)st100.62$, the dominant enhancement of the Abl mutant phenotype reported for $Df(3L)st4$ (Henkemeyer et al., 1987) is likely to be due to its removal of the fax gene, distal to Abl (Fig. 5). However, Henkemeyer et al. (Henkemeyer et al., 1987) have also shown that $Df(3L)st-e5$ is a strong dominant enhancer of the Abl mutant phenotype. Based on its reported cytology, $Df(3L)st-e5$ is wild-type for fax (Fig. 5). The work of de la Escalera et al. (de la Escalera et al., 1990) has shown the $Df(3L)st-e5$ chromosome is wild type for nrt. Thus, this deficiency apparently uncovers another gene in the 73A-C region that can dominantly enhance the Abl mutant phenotype. We are currently pursuing the identity of this gene.

This report is not the first description of genetic interactions between Abl and genes encoding cell adhesion molecules. Similar to the strong phenotypic effects on CNS architecture that we have described in animals homozygous mutant for Abl and nrt (Fig. 3C,F), Elkins et al. (Elkins et al., 1990) have found that animals homozygous mutant for both Abl and fasciclin I (fasI) show a mutant phenotype affecting midline crossing of commissural axons. Unlike the present study, however, heterozygous mutations in fasI had no effect on the Abl mutant phenotype. The discovery of dosage-sensitive genetic interactions, such as that between Abl and nrt, may indicate that the proteins encoded by these two genes are biochemically linked. The continued search for dosage-sensitive modifiers of the Abl mutant phenotype, and the continued biochemical characterization of these modifiers will undoubtedly deepen our picture of the molecular machinery guiding neuronal growth cones.

R.G.R., A.L.S. and E.R.S. were supported in part by the Anderson Summer Research Assistantship Fund of Denison University. We are grateful to J. Terman and A. Kolodkin for their initial assistance with RNAI experiments, and to F.M. Hoffmann and P. Kuhlman for critical reading of the manuscript. This work was supported by National Institutes of Health grant R15-CA70890 and funds from the Denison University Research Foundation to E.C.L., and National Science Foundation grant IBN-0090239 to E.C.L. and M.A.S.

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