The Derailed Guidance Receptor Does Not Require Kinase Activity *In Vivo*

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The *Drosophila* Derailed (DRL) receptor tyrosine kinase (RTK) controls key guidance events in the developing nervous system and mesoderm. Like other members of the "related to tyrosine kinases" (RYK) subfamily of RTKs, DRL has several highly unusual amino acid substitutions within the catalytic domain, raising the possibility that members of this subfamily are catalytically inactive. To test the role of DRL kinase activity *in vivo*, we mutated the invariant lysine required for catalytic activity of known kinases and examined the ability of this mutant to function in two assays: a dominant gain-of-function axon switch assay in the nervous system and phenotypic rescue of muscle attachment in *drl* mutants. We show that this predicted kinase-deficient DRL mutant is capable of functioning in both assays. Our results indicate that DRL does not require kinase activity *in vivo* and suggest that members of the RYK subfamily of RTKs transduce signals unconventionally.

Key words: receptor tyrosine kinase; kinase; Drosophila*; axon guidance; Derailed; midline*

Many of the cell–cell interactions underlying morphological events during development involve the transduction of extracellular signals by receptor tyrosine kinases (RTKs). RTKs dimerize upon ligand binding, resulting in autophosphorylation and subsequent recruitment of cytoplasmic signaling components involved in the cellular response. The *Drosophila* Derailed (DRL) RTK controls key events in the differentiation of both the embryonic CNS and mesoderm (Callahan et al., 1995, 1996; Bonkowsky et al., 1999) and is required for establishing the normal morphology of the adult CNS (Moreau-Fauvarque et al., 1998; Simon et al., 1998).

DRL is a member of the "related to tyrosine kinases" (RYK) subfamily of RTKs that includes mammalian RYK, a single *Caenorhabditis elegans* member, and two additional members in *Drosophila* (Hovens et al., 1992; Callahan et al., 1995; Oates et al., 1998; Halford et al., 1999; Savant-Bhonsale et al., 1999) (S. Yoshikawa and J. B. Thomas, unpublished observations). Within the cytoplasmic domain, all RYK subfamily members contain the 11 subdomains that are hallmarks of the broad family of protein kinases (Hanks et al., 1988). Furthermore, each member has the invariant lysine of subdomain II that is essential for the phosphotransfer reaction of active kinases (Carrera et al., 1993). However, all subfamily members share several unusual amino acid substitutions in regions of the catalytic domain that are normally highly conserved in other RTKs (Hanks et al., 1988). The most notable of these are substitution of the first glycine within the subdomain I nucleotide binding motif [(Q/M/K)XGXXG for GXGXXG] and substitutions within the canonical activation loop motif of subdomain VII [D(S/N)(A/S) for DFG].

These unusual amino acid substitutions have raised the possibility that members of this RTK subfamily are catalytically inactive. Arguing against catalytic activity is the finding that autophosphorylation of a TrkA extracellular/RYK cytoplasmic chimeric receptor expressed in cell culture is undetectable in response to the TrkA ligand NGF, despite the ability of the chimera to activate the MAP kinase cascade under the same conditions (Katso et al., 1999). Restoring the canonical DFG motif to the atypical RYK activation loop sequence results in the ability of the TrkA/RYK chimera to autophosphorylate (Katso et al., 1999). Together with the lack of activity for RYK in an *in vitro* kinase assay (Hovens et al., 1992), these results suggest that RYK subfamily members might normally be catalytically inactive. However, mutating the invariant lysine of subdomain II of the RYK catalytic domain abolishes the capacity of the TrkA/RYK chimera to activate the MAP kinase pathway, suggesting that RYK may normally be capable of low levels of catalytic activity sufficient to activate the MAPK pathway (Katso et al., 1999). How these observations might relate to the *in vivo* function of RYK in particular, and the RYK subfamily of RTKs in general, is not known. To address this, we have tested *in vivo* a DRL mutant protein predicted to be catalytically inactive and show that it retains function in two separate assays, thus providing the first

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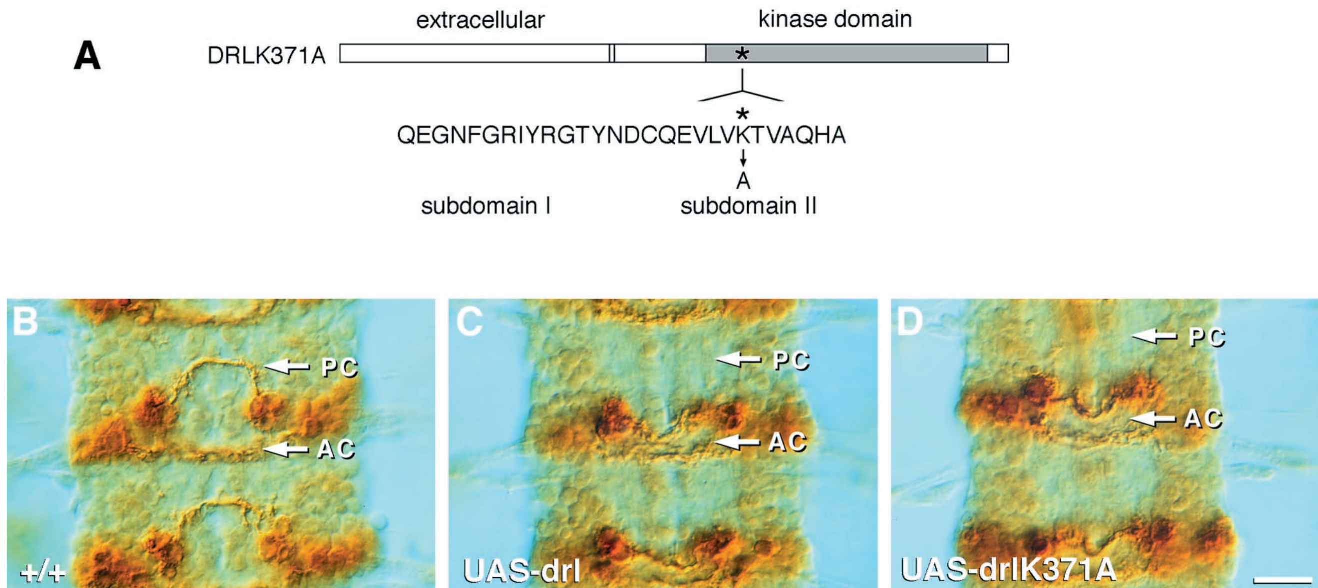

Figure 1. *In vivo* axon switching ability of DRL and DRLK371A in the CNS of *Drosophila* embryos. *A*, Schematic of DRLK371A, a predicted kinase-dead mutant derivative tested for activity *in vivo*. Wild-type DRL and DRLK371A were each cloned downstream of GAL4 binding sites (*UAS*) or the *apterous* muscle enhancer ME4 (Callahan et al., 1996), enabling cell-specific expression of these transgenes in the CNS or muscles, respectively. DRL amino acid sequence shows the lysine-to-alanine mutation at position 371 (*asterisk*) in subdomain II. *B–D*, Embryos carrying the *eg-GAL4* driver plus *UAS* transgenes denoted. *B*, The two clusters of Eg neurons extend axons across the midline as assayed by staining for Tau-β-gal reporter expressed from a *UAS-tau-lacZ* reporter transgene. Each cluster forms a distinct axon bundle, one in the anterior commissure (*AC*) and the other in the posterior commissure (*PC*). *C*, When forced to misexpress DRL from three copies of a *UAS-drl* transgene, all of the Eg PC axons switch their projections and cross the midline in the AC, forming a bundle distinct from the normal Eg AC bundle. *D*, Misexpression of DRLK371A from three copies of a *UAS-drlK371A* transgene switches PC axons to the AC in 96% of segments. Scale bar, 20 μm. See Table 1 for quantification of the data.

evidence that catalytic activity is not required *in vivo* for function of an RYK subfamily member.

MATERIALS AND METHODS

DNA constructs. Manipulations of the *drl* cDNA are based on the published full-length cDNA clone (Callahan et al., 1995). To generate pBS-drlK371A, a fragment (nucleotide 1268–1668) carrying a site-directed mutagenesis (Ho et al., 1989) of lysine to alanine at aa 371 (-tca-aga- to -tcg-cga-) was cloned into the corresponding *Nde*I/*BsiW*I of pBS-drl. pME4-drlK371A was created by cloning the blunted *Eco*RI fragment from pBS-drlK371A into the blunted *Xba*I site of pME4. pUAS-drlK371A was made by cloning the *Eco*RI fragment into pUAST. All PCR-amplified products and cloning junctions were sequenced. Restriction enzyme analysis of transgenic flies confirmed that *UAS-drlK371A* and *ME4-drlK371A* had been inserted in transformants (a novel *Nru*I site is created by the nucleotide change).

Fly strains and genetics. P element transformation was performed using standard methods (Rubin and Spradling, 1982). For each transgene, multiple lines were generated and checked for expression. Lines were homozygosed if viable and fertile or balanced over *FM7c*, *CyO*, or *TM3*. When necessary, *CyO,wg-lacZ* or *TM3,ftz-lacZ* balancer chromosomes were used to independently score the genotypes of embryos. All fly crosses and embryo collections were performed at 25°C.

Immunohistochemistry. Embryo dissections, phalloidin staining, and HRP immunostainings were performed as described previously (Callahan and Thomas, 1994; Callahan et al., 1996; Thor and Thomas, 1997).

RESULTS

The DRLK371A mutant receptor

To test whether kinase activity of DRL is required for function *in vivo*, we compared the activity of full-length DRL with DRLK371A. To generate DRLK371A, we mutated the invariant lysine of Subdomain II at position 371 to an alanine (Fig. 1*A*). Because this lysine is involved in the phosphotransfer reaction and has been shown to be essential for catalytic activity of tyrosine kinases (Carrera et al., 1993), DRLK371A is predicted to be catalytically inactive. By expressing DRLK371A in a *drl* mutant background and staining embryos with an anti-DRL antibody, we found it to be localized to growth cones and axons of neurons and to the growing tips of muscles, similar to the localization of full-length DRL expressed under the same conditions (Callahan et al., 1996) (data not shown).

DRLK371A switches axons from the posterior commissure to the anterior commissure

In the embryonic CNS, DRL is expressed on the growth cones and axons of all neurons that traverse the midline in the anterior commissure (AC), one of two tracts in each segment that connect opposite sides of the bilaterally symmetric *Drosophila* nervous system (Callahan et al., 1996). In mutants lacking DRL, AC neurons abnormally cross in the posterior commissure (PC). Conversely, misexpression of DRL in PC neurons switches their axonal projections to the AC (Bonkowsky et al., 1999). This pathway-switching behavior of DRL-misexpressing neurons and the *in vivo* binding pattern of a soluble DRL receptor probe indicate that DRL acts as a guidance receptor for an unknown repellent ligand present in the PC (Bonkowsky et al., 1999).

Table 1. PC to AC axon switching by DRL and DRLK371A

	Percentage of segments with:		
Genotype[a]	All axons switched	Some axons switched	No axons switched
Wild type	0	0	100 (n = 100)
UAS-drl (3 copies)	99	1	0 (n = 103)
UAS-drlK371A (3 copies)	82	14	4 (n = 77)

[a] All genotypes contain *eg-GAL4* plus a single copy of a *UAS-tau-lacZ* reporter transgene to visualize the axons of expressing neurons.

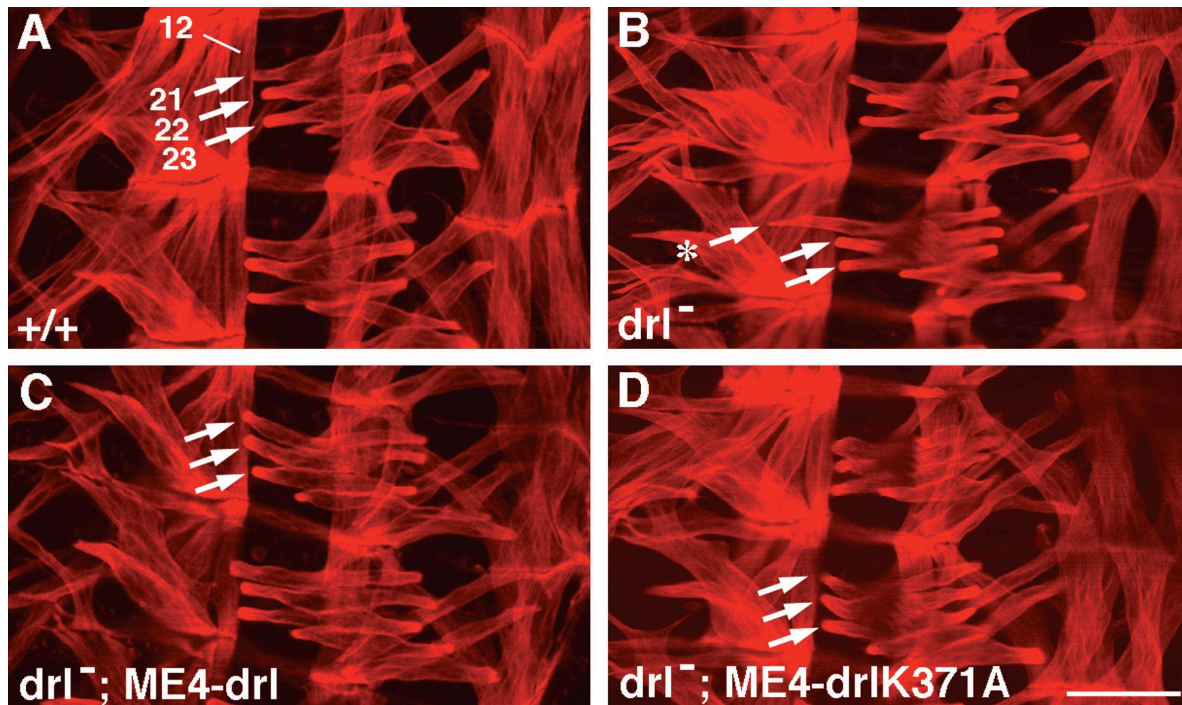

Figure 2. DRL and DRLK371A function in muscle attachment. Embryos were incubated with RITC-conjugated phalloidin, which stains all muscles. Two hemisegments are shown in each *panel*; anterior is *up*, ventral is *left*. The ventral epidermal insertion sites of muscles 21–23, which normally express DRL, are denoted by *arrows*. *A*, In wild type, muscles 21–23 always insert near the dorsal border of muscle 12. *B*, In *drl* ^{R343} null mutant embryos, muscles of the 21–23 group bypass their normal insertion sites near muscle 12 and project more ventrally in ~20% of hemisegments (*asterisk* denotes abnormal ventral insertion of muscle 21). *C*, *D*, Two copies of *ME4-drl* (*C*) or *ME4-drlK371A* (*D*) completely rescue the *drl* muscle bypass phenotype. Scale bar, 40 μm. See Table 2 for quantification of the data.

Table 2. Rescue of the *drl* muscle phenotype by DRL and DRLK371A

Genotype	Percentage of hemisegments with one or more "bypass" muscles
Wild type	0 (n = 117)
drl^{R343}	19 (n = 118)
drl^{R343}; *ME4-drl*	0 (n = 116)
drl^{R343}; *ME4-drlK371A*	0 (n = 115)
drl^{R343}; *ME4-lacZ*	23 (n = 114)

We compared the ability of DRLK371A and DRL to switch the commissure choice by targeting their expression to PC neurons using the GAL4/UAS transactivation system (Brand and Perrimon, 1993). *eagle-GAL4* (*eg*-GAL4) (Dittrich et al., 1997) is a P[GAL4] line that drives expression in such a sufficiently small subset of PC neurons that we could unambiguously follow their axonal projections (Bonkowsky et al., 1999). As assayed with an axon-targeting *UAS-tau-lacZ* reporter transgene (Callahan and Thomas, 1994), *eg*-GAL4 drives expression in two small clusters of interneurons in each hemisegment (Fig. 1*B*). One cluster projects axons across the midline in the PC and the other in the AC. The axons from homologous clusters on either side of the midline fasciculate with one another, forming two distinct bundles of axons, one within each of the commissures. When forced to misexpress wild-type DRL using three copies of a *UAS-drl* transgene, PC neurons switch their axon projections to the AC in 100% of segments (Fig. 1*C*, Table 1). To assay the requirement of an active kinase domain, we misexpressed DRLK371A from three copies of a *UAS-drlK371A* transgene. We found that 96% of segments showed some or all axons switching from the PC to the AC, indicating that an active kinase domain is not required for DRL function in switching PC neurons to the AC (Fig. 1*D*, Table 1). We previously showed that the ability of DRL to switch axons is dose dependent (Bonkowsky et al., 1999). Thus, the observation that misexpression of DRLK371A results in fewer segments with all axons switched (82 vs 99%) most likely reflects a difference between the levels of expression of three copies of *UAS-drlK371A* and *UAS-drl*.

DRLK371A rescues muscle attachment defects in *drl* mutants

DRL plays a guidance role in the mesoderm where it is required for a subset of muscles to halt their extension and make stable attachments to their appropriate epidermal tendon cells (Callahan et al., 1996). The somatic musculature of the *Drosophila* embryo consists of a stereotyped arrangement of large multinucleate muscle fibers with predictable orientations and attachment sites. Developing muscles extend growth cone-like processes, which lead a migration along the epidermis. DRL is expressed by muscles 21–23 as they grow toward and attach to their set of specialized epidermal cells. Normally, muscles 21–23 extend similar distances ventrally and always attach to their epidermal attachment cells near muscle 12 (Fig. 2*A*). In *drl* null mutants such as *drl* ^{R343}, ~20% of hemisegments contain one or more muscles of the 21–23 group that bypass their correct epidermal attachment sites and instead attach to inappropriate sites located more ventrally (Callahan et al., 1996) (Fig. 2*B*, Table 2). This bypass phenotype can be completely rescued by targeted expression of wild-type DRL using the muscle enhancer *ME4* to drive expression of a *drl* cDNA in these muscles (Fig. 2*C*, Table 2) (Callahan et al., 1996).

We capitalized on this rescuing assay to test the requirement of

DRL kinase activity. We generated transformants carrying *ME4-drlK371A* to express this mutant variant in the DRL muscles. No effect of either transgene on muscle development was observed in a wild-type background (data not shown), indicating that similar to what we observed for axon switching in the CNS, DRLK371A does not interfere in a dominant-negative fashion with normal DRL function.

In a *drl*^{R343} mutant background, two copies each of either *ME4-drl* or *ME4-drlK371A* were sufficient to completely rescue the mutant muscle phenotype (Fig. 2*D*, Table 2). As a control we tested a *ME4-lacZ* transgene and found that it failed to show any rescue (Table 2). Thus, the rescuing ability of *ME4-drl* and *ME4-drlK371A* is dependent on the DRL sequences within the transgenes. From these results we conclude that catalytic activity is not required for DRL function in muscle attachment.

DISCUSSION

Using two *in vivo* assays, we have shown that a predicted catalytically dead mutant of DRL is capable of functioning at levels comparable with wild type. For these studies we used a dominant gain-of-function assay in the CNS and a phenotypic rescuing assay in the mesoderm. Ideally, we would like to have been able to assess DRL function in a rescuing assay in the CNS. However, at present we do not have the ability to express DRL exclusively in AC neurons (i.e., the DRL neurons) in a *drl* null mutant background, a necessary requirement for such an assay because ectopic expression of DRL causes dominant PC to AC switching.

Consistent with our results are the unusual amino acid substitutions within the kinase domain and the lack of demonstrable kinase activity *in vitro* and in cell culture for members of the RYK subfamily (Hovens et al., 1992; Wang et al., 1996; Katso et al., 1999). Our results strongly suggest that DRL, and by extension other members of the RYK subfamily of RTKs, transduces signals *in vivo* independent of their catalytic activity. Transduction of DRL signals might involve the formation of heterodimers with another active RTK, in which case its signaling could be dependent on transphosphorylation of its cytoplasmic domain by this partner. A precedent for this scenario is the formation of heterodimers between the catalytically inactive ErbB3 RTK and other active members of the ErbB subfamily (Carraway and Cantley, 1994). An alternative mode for DRL action could involve the recruitment of signaling components independent of its phosphorylation state. For example, ligand binding might induce a conformational change of the DRL cytoplasmic domain that would subsequently allow binding of the appropriate signaling components. In either event, our *in vivo* results indicate that DRL and possibly the entire RYK subfamily of atypical RTKs transduce signals in an unconventional manner.

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