Src family kinases are required for WNT5 signaling through the Derailed/RYK receptor in the Drosophila embryonic central nervous system

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Members of the RYK/Derailed family have recently been shown to regulate axon guidance in both Drosophila and mammals by acting as Wnt receptors. Little is known about how the kinase activity-deficient RYKs transduce Wnt signals. Here, we show that the non-receptor Src family tyrosine kinases, SRC64B and SRC42A, are involved in WNT5-mediated signaling through Derailed in the Drosophila embryonic central nervous system. Analysis of animals lacking SRC64B and SRC42A reveals defects in commissure formation similar to those observed in Wnt5 and derailed mutants. Reductions in SRC64B expression levels suppress a Wnt5/derailed-dependent dominant gain-of-function phenotype, and increased levels of either SRC64B or SRC42A enhance Wnt5/derailed-mediated axon commissure switching. Derailed and SRC64B form a complex, which contains catalytically active SRC64B, the formation or stability of which requires SRC64B kinase activity. Furthermore, Derailed is phosphorylated in a SRC64B-dependent manner and coexpression of Derailed and SRC64B results in the activation of SRC64B. The mammalian orthologs of Derailed and SRC64B also form complexes, suggesting that Src roles in RYK signaling are conserved. Finally, we show that coexpression of WNT5 and Derailed has no apparent effect upon TCF/LEF-dependent transcription, suggesting that the Src family kinases play novel roles in WNT5/Derailed-mediated signaling.

KEY WORDS: Axon guidance, RYK, Src family kinase, Wnt, Signal transduction

INTRODUCTION
During the development of the nervous system, axons are guided by attractive and repulsive guidance cues (Dickson, 2002). The ventral midline of the Drosophila embryonic nervous system has proven to be an excellent model system in which to identify the molecules that control axon guidance (Araujo and Tear, 2003). A number of these guidance proteins are encoded by highly conserved gene families; for example, slit, roundabout and netrin genes have been shown to play remarkably similar roles to their Drosophila orthologs at the mammalian floorplate (Garbe and Bashaw, 2004).

Whereas considerable knowledge has accumulated about the mechanisms controlling initial midline crossing, less is known about those controlling routing at intermediate choice points, where extending axons may take alternative routes. An example of such a decision is commissure choice. Drosophila contralateral axons project stereotypically through one of the two major axon tracts in each hemisegment, the anterior (AC) or the posterior (PC) commissure. An axon’s projection through the AC is, at least in part, dictated by its repulsion away from the PC by the Wnt family member WNT5 acting through the Derailed (DRL) RYK axonal receptor (Bonkowski et al., 1999; Callahan et al., 1995; Yoshikawa et al., 2003). Wnt5 (Fradkin et al., 2004) and drl (Callahan et al., 1995) mutants also display altered axon fasciculation, which might reflect changes in inter-axonal adhesion. Moreover, mutation of drl (also known as linotte) results in memory deficits (Dura et al., 1993), likely to be caused by axon guidance defects in the larval brain (Moreau-Fauvarque et al., 1998; Simon et al., 1998).

Wnt family proteins signal through alternative receptors with distinct downstream pathways that sometimes have members in common. In many tissues, Wnts signal by binding to the Frizzled (Fz) family of receptors in conjunction with LRP co-receptors (Cadigan and Nusse, 1997). Fzs can transduce Wnt signaling via a canonical Armadillo/β-catenin pathway culminating in the regulation of TCF/LEF-dependent transcription or via non-canonical pathways (Widelitz, 2005), some involving the heterotrimeric GTPases (Katanaev et al., 2005; Katanaev and Tomlinson, 2006; Liu et al., 2001). Recently, the mammalian WNT5A protein was shown to interact with the receptor tyrosine kinase (RTK) ROR, resulting in the repression of canonical Wnt signaling via an as yet uncharacterized mechanism (Mikels and Nusse, 2006). In these studies, WNT5A was also shown to activate the canonical Wnt signaling pathway via interaction with a Fz family member, suggesting that pathway specificity might sometimes be determined by the Wnt receptor engaged and not solely by the specific Wnt itself.

In addition to their involvement in a number of diverse developmental processes (Logan and Nusse, 2004), Wnts play roles in various aspects of nervous system development, such as cell fate determination, synapse formation, axon guidance and neurite outgrowth (Ciani and Salinas, 2005; Fradkin et al., 2005; Zou, 2004). Wnt-RYK interactions (reviewed by Bovolenta et al., 2006; Keeble and Cooper, 2006) underlie the anterior-posterior guidance of subsets of axons in the mammalian spinal cord (Liu et al., 2005), cortical axon guidance across the corpus callosum (Keeble et al., 2006), establishment of the vertebrate retinotectal topographic map (Schmitt et al., 2005) and neurite outgrowth in vivo and in cultured primary cells (Lu et al., 2004).
Members of the RYK family of ‘dead’ or ‘fractured’ RTKs have been found in all metazoans examined (Halford and Stacker, 2001). The extracellular domain of RYK members contains a Wnt-binding WIF domain (Pathly, 2000). RYKs bear substitutions in highly conserved amino acid residues required for phosphotransfer that are likely to render them inactive as kinases. Although apparently lacking kinase activity, a human TRKA (NTRK1)-RYK fusion protein was shown to activate the MAPK pathway when bound by NGF (Katso et al., 1999), suggesting that RYKs transduce extracellular signals to downstream targets within the cell. Recent studies (Grillenzoni et al., 2007; Yao et al., 2007) have shown that DRL can also act to antagonize WNT5 and WNT5 expression plasmids were constructed by ORF PCR, oligonucleotide-mediated mutagenesis and Gateway-mediated recombination (Invitrogen) into appropriate destination vectors (provided by T. Murphy; http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). Y. Zou and K-L. Guan, respectively, provided HA-tagged mouse RYK (Liu et al., 2005) and human c-SRC (Li et al., 2004) expression plasmids. The WUS-WRAPPER construct (Noordermeer et al., 1998) was co-transfected with pAc-GAL4 to express Wraper. To generate an Src42B-specific RNA interference transgene, gene-specific inverted repeats (bp 1363 to 1963 of accession number NM_080195) were cloned into a pPLUST derivative bearing an intervening intron. Decreases in Src42B mRNA levels were determined by semi-quantitative reverse transcribed (RT)-PCR of first strand embryonic cDNA as described (see Fig. S1 in the supplementary material). Similar reductions in Src42B expression were observed with two different inserts. All plasmids were verified by sequencing.

S2 and Kc cell transfections were performed using Effectene (Qiagen) and 293T cell transfections with Fugene (Roche). Lysates were prepared using a high-stringency SDS-containing RIPA buffer (Muda et al., 2002) containing a cocktail of protease inhibitors (Roche). Drosophila cell lysate immunoprecipitations were performed using rabbit anti-Myc (Upstate) or mouse anti-rabbit light chain mAb (Jackson Immunoresearch) was used to reduce the recognition of the rabbit anti-DRL antibodies used in immunoprecipitations on blots probed with rabbit antisera. For the double immunoprecipitation of DRL to assess its phosphorylation content, lysates were first precipitated with anti-DRL, washed immune complexes boiled in 1% SDS, diluted 1:10 into buffer containing 1% Triton X-100 and then DRL-HA immunoprecipitated with reagents from the Super8XTop/FopFlash plasmids ([Veenam et al., 2003]; a kind gift from R. Veenam).
Fig. 1. SFKs play redundant roles during formation of the embryonic CNS commissures. Stage 16 Drosophila embryos of the indicated genotypes were stained with mAb BP102 to label all central axons. Anterior is up. (A) Wild type, (B) Wnt5\textsuperscript{G001}, (C) Src42A\textsuperscript{E1}, (D) Src64B\textsuperscript{KO}, (E) Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO}, (F) Src42A\textsuperscript{E1}, Src64B\textsuperscript{KO}, (G) Src42A\textsuperscript{E1}, Src64B\textsuperscript{KO}, (H) Src42A\textsuperscript{E1}, Src64B\textsuperscript{KO}, (I) ELAV-GAL4/UAS-RNAi-Src64B and (J) Src42A\textsuperscript{E1}, ELAV-GAL4/UAS-RNAi-Src64B are shown. Defects similar to those seen in Wnt5\textsuperscript{G001}, namely ‘fuzzy’ commissures and breaks in the longitudinal pathways, are observed in individuals homozygous for one of the SFK mutants and heterozygous for the other and also in individuals of a Src42A mutant background when Src64B expression is reduced in the nervous system by RNA interference. The commissures are completely fused (white arrow in H) in the double homozygotes. See Table 1 for quantitation. AC, anterior commissure; PC, posterior commissure.

RESULTS
SFKs are required for embryonic commissure formation

To identify members of the Wnt/drl signaling pathway in Drosophila, we analyzed candidate proteins in a directed yeast two-hybrid approach. Src64B, a member of the non-receptor tyrosine kinase SFK family (Thomas and Brugge, 1997), was found to interact with the DRL intracellular domain bait fusion protein (data not shown). In order to evaluate possible roles of Src64B in Wnt5/drl-mediated signaling, we examined the ventral nerve cord commissures in embryos lacking Src64B, Src42A, or both. Homozygosity for null alleles of either Src64B or Src42A or simultaneous heterozygosity for both Src64B and Src42A resulted in only mild aberrations in commissural projections (Fig. 1C-E). However, embryos entirely lacking one of the SFK orthologs and heterozygous for a null allele of the other (Fig. 1F,G) displayed ‘fuzzy’ commissures, longitudinal breaks and apparent axon stalling in the longitudinal pathways, as previously reported for Wnt5-null mutant embryos (Fradkin et al., 2004) (Fig. 1B). Embryos entirely lacking both Src64B and Src42A displayed a highly penetrant, severe commissural phenotype (Fig. 1H). Embryos lacking SRC42A and pan-neuronally expressing Src64B-targeting double-stranded (ds) RNA (Fig. 1I, Table 1) displayed qualitative and quantitative phenotypes similar to those of Src42A, Src64B\textsuperscript{KO} and Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO} mutant embryos. This result confirms a requirement for wild-type Src64B expression levels in SRC42A-deficient neurons for commissure formation. Altered commissure formation in the partial SFK mutants is unlikely, therefore, to be due to secondary effects of the loss of Src64B in other tissues. The failure of these animals to exhibit the full SFK-null commissural phenotype probably reflects incomplete knockdown of Src64B expression levels (see also below). Quantitation of these phenotypes is presented in Table 1. These results and the similar commissural phenotypes observed in a previous study examining SFK roles during Drosophila embryonic development (Takahashi et al., 2005) suggest that the Drosophila SFKs play partially redundant roles in the formation of the embryonic commissures.

Table 1. Abnormal commissural axonal projections in SFK mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Segments with abnormal commissural axonal projections (%)</th>
<th>Number of segments scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>(w^{118})</td>
<td>0</td>
<td>251</td>
</tr>
<tr>
<td>Wnt5\textsuperscript{G001}</td>
<td>67</td>
<td>237</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}</td>
<td>11.5</td>
<td>330</td>
</tr>
<tr>
<td>Src64B\textsuperscript{KO}</td>
<td>12.5</td>
<td>350</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO}</td>
<td>6.4</td>
<td>280</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO}</td>
<td>39</td>
<td>270</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO}</td>
<td>26</td>
<td>180</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}; Src64B\textsuperscript{KO}</td>
<td>99</td>
<td>150</td>
</tr>
<tr>
<td>ELAV-Gal4/UAS-RNAi-Src64B</td>
<td>12</td>
<td>449</td>
</tr>
<tr>
<td>Src42A\textsuperscript{E1}; ELAV-Gal4/UAS-RNAi-Src64B</td>
<td>33</td>
<td>496</td>
</tr>
</tbody>
</table>

Embryos of the indicated genotypes were scored for thinning or loss of the commissures after staining with the pan-axonal BP102 monoclonal antibody.
We also generated embryos that have reduced levels of SRC64B in a lineage that crosses in the AC, the commissure most affected by the absence of del or Wnt5, by expressing Src64B-specific dsRNA under control of a SEMA2B-GAL4 driver (Materials and methods) (Brand and Perrimon, 1993). Sema2b is expressed in a small subset of segmentally reiterated neurons that project their axons to the contralateral side through the AC (Rajagopalan et al., 2000). Src64B mRNA levels in dsRNA-expressing animals were evaluated by semi-quantitative RT-PCR and found to be ~10% of wild-type levels (see Fig. S1A in the supplementary material). Sema2b+ axons with decreased levels of Src64B misprojected or apparently stalled in a number of hemisegments (25%, n=383) (see Fig. S1B in the supplementary material). No apparent changes in Sema2b+ cell fate or cell body position were observed. These data provide further support for SFK roles in commissure formation.

To address whether there were widespread CNS patterning defects in SFK mutant animals that might cause the aberrant commissure formation, we examined the midline and lateral glia in the various SFK mutant combinations by staining with anti-Wrapper (Noordermeer et al., 1998) and anti-REPO (Alonso and Jones, 2002) antibodies. The lateral glial cells appeared in their wild-type positions in all mutant combinations except the Src42A+/-; Src64B+/- and double-null mutants, whereas the midline glia appeared wild-type in all combinations except the double-null SFK mutant (see Fig. S2 in the supplementary material). The intermediate commissural phenotypes seen in the ‘partial’ SFK mutants are therefore unlikely to be caused by major CNS patterning and fate changes.

Wild-type Src64B expression levels are required for Wnt5/drl-mediated axon repulsion

Next we examined whether Src64B genetically interacts with the Wnt5/drl-mediated signaling pathway during embryonic nervous system development. We first evaluated whether wild-type Src64B levels were required for a previously reported Wnt5 dominant gain-of-function phenotype (Fradin et al., 2004; Yoshikawa et al., 2003). When Wnt5 was ectopically expressed from a single transgene in the midline glia using the SIM-GAL4 driver, ~16% of hemisegments displayed the absence or thinning of the AC (Fig. 2A, Table 2), owing to the repulsion of the DRL+ AC axons by WNT5 produced by the midline glia. This assay was previously used to establish that wild-type expression levels of the O-acyltransferase porcupine (por; por – FlyBase) (Fradkin et al., 2004) and of del (Yoshikawa et al., 2003) are required for the loss of the AC, thus confirming that these genes are members of a WNT5 signaling pathway. The removal of only a single copy of Src64B from this genetic background resulted in a greater than 5-fold reduction in the loss of the AC (Fig. 2, compare A and B; Table 2). The extent of suppression observed in Src64B heterozygotes was similar to that seen in animals heterozygous for del (Table 2). Heterozygosity for Src42A, by contrast, did not suppress the WNT5-dependent midline overexpression phenotype (Table 2). These data indicate that wild-type Src64B expression levels are required for this dominant gain-of-function phenotype and that Src64B is therefore likely to be a member of the Wnt5/drl signaling pathway.

Table 2. Heterozygosity for Src64B suppresses the Wnt5 midline glial overexpression phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Loss or thinning of AC (%)</th>
<th>Loss or thinning of PC (%)</th>
<th>Number of segments scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118</td>
<td>0</td>
<td>0</td>
<td>251</td>
</tr>
<tr>
<td>sim-Gal4, UAS-WNT5/+</td>
<td>16</td>
<td>0</td>
<td>240</td>
</tr>
<tr>
<td>sim-Gal4, UAS-WNT5/+; src64b/-/+</td>
<td>3</td>
<td>0</td>
<td>264</td>
</tr>
<tr>
<td>sim-Gal4, UAS-WNT5/+; src42a–/+</td>
<td>17</td>
<td>0</td>
<td>256</td>
</tr>
<tr>
<td>sim-Gal4, UAS-WNT5/+; src42a–/+</td>
<td>3</td>
<td>0</td>
<td>248</td>
</tr>
<tr>
<td>porc/Y; sim-Gal4, UAS-WNT5/+</td>
<td>2</td>
<td>0</td>
<td>380</td>
</tr>
</tbody>
</table>

Embryos of the indicated genotypes were scored for thinning or loss of the commissures after staining with the pan-axonal BP102 monoclonal antibody.
Enhancement of switching was also observed when DRL-MYC; H11003 overlapped with mRNA was observed throughout the ventral nerve cord (Fig. 3A) and relative to those of neurons, we determined the expression domains of support the hypothesis that the SFKs are members of the SRC64B is required for its interaction with DRL (Table 3). These data did not increase switching, indicating that the kinase activity of University of Rochester, Rochester, NY) in this sensitized background expression of wild-type Src64B in EG-GAL4+ axons in the sensitized background (single copy of UAS-DRL-MYC) resulted in significant enhancement in the number of switched axons (1% and 34% switched axons for 1 × UAS-DRL-MYC; 1 × UAS-NLS β-Gal and 1 × UAS-DRL-MYC; 1 × UAS-SRC64B, respectively; Fig. 2E, Table 3). Enhancement of switching was also observed when Src42A expression levels were increased, but to a lesser extent than with overexpression of SRC64B (8% versus 34%, respectively; Table 3). Expression of a kinase-deficient Src64B transgene (gift of Willis Li, University of Rochester, Rochester, NY) in this sensitized background did not increase switching, indicating that the kinase activity of SRC64B is required for its interaction with DRL (Table 3). These data support the hypothesis that the SFKs are members of the Wnt5/drl-signaling pathway.

**SRC64B and DRL are both expressed in AC axons**

To determine whether Src64B and drl are normally coexpressed in neurons, we determined the expression domains of Src64B mRNA relative to those of Wnt5 and drl in the embryonic CNS. Src64B mRNA was observed throughout the ventral nerve cord (Fig. 3A) and overlapped with drl mRNA in the anterior part of each segment (Fig. 3B). drl RNA is expressed in neuronal cell bodies that send their projections through the AC (Bonkowsky et al., 1999; Callahan et al., 1995; Yoshikawa et al., 2003), whereas Wnt5 mRNA is most predominantly found in neuronal cell bodies associated with the PC (Fig. 3C) (Fradkin et al., 2004). SRC64B protein was found in most, if not all, longitudinal and commissural axonal projections through the AC (Bonkowsky et al., 1999; Callahan et al., 1995). SRC42A and DRL are therefore expressed in AC axonal projections, supporting our observations that they functionally interact there.

**SRC64B and DRL and their mammalian orthologs physically interact**

We then evaluated whether SRC64B and DRL physically interact by ascertaining their ability to co-immunoprecipitate from transiently transfected tissue culture cell lysates. We expressed

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PC to AC switching (%)</th>
<th>No axons switching (%)</th>
<th>Number of segments scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × UAS-mCD8-GFP</td>
<td>0</td>
<td>100</td>
<td>567</td>
</tr>
<tr>
<td>2 × UAS-DRL-MYC</td>
<td>56</td>
<td>44</td>
<td>550</td>
</tr>
<tr>
<td>1 × UAS-DRL-MYC; 1 × UAS-NLS β-Gal</td>
<td>1</td>
<td>99</td>
<td>459</td>
</tr>
<tr>
<td>1 × UAS-DRL-MYC; 1 × UAS-SRC64B</td>
<td>34</td>
<td>66</td>
<td>510</td>
</tr>
<tr>
<td>1 × UAS-mCD8-GFP; 1 × UAS-SRC64B</td>
<td>0</td>
<td>100</td>
<td>418</td>
</tr>
<tr>
<td>1 × UAS-DRL-MYC; 1 × UAS-SRC42A K312R</td>
<td>3</td>
<td>97</td>
<td>566</td>
</tr>
<tr>
<td>1 × UAS-DRL-MYC; 1 × UAS-SRC42A</td>
<td>8</td>
<td>92</td>
<td>420</td>
</tr>
</tbody>
</table>

All embryos carry, besides the listed chromosomes, the EG-Gal4 insert, which was present in single copy in all genotypes except the 2 × UAS-DRL-MYC where it was present in two copies. EG-Gal4+ axons were visualized using anti-MYC and scored for PC to AC switching when UAS-Drl-myc was present or by use of anti-GFP for UAS-mCD8-GFP.

Fig. 3. Src64B mRNA expression overlaps with drl mRNA expression and SRC64B protein is present in axons. (A) Wild-type Drosophila embryo labeled with a Src64B antisense RNA probe shows Src64B expression throughout the ventral nerve cord (arrow) and in the gut. (B) Double RNA in situ staining for endogenous Src64B mRNA (green) and drl mRNA (red) shows that drl and Src64B overlap in the ventral nerve cord in the anterior portion of each segment. (C) Double RNA in situ staining for endogenous Wnt5 mRNA (green) and drl mRNA (red) shows that Wnt5 is predominantly expressed in PC-associated neuronal cell bodies that do not express drl. (D) SRC64B protein is expressed in the wild-type longitudinal and commissural axons. (E) Axons of a homozygous Src64B<sup>−/−</sup> mutant embryo are not stained by anti-SRC64B. (F) SRC64BPI-ss RNA in situ staining for endogenous Wnt5 mRNA (green) and drl mRNA (red) shows that Wnt5 mRNA is most predominantly expressed in PC-associated neuronal cell bodies that do not express drl.
RAPID COMMUNICATION

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Epitope-Tagged DRL and SRC64B Proteins in Drosophila Kc Cells

The expression of DRL and SRC64B was confirmed by immunoblotting of the whole-cell extract (WCE). DRL and SRC64B specifically co-immunoprecipitate in the presence or absence of WNT5 protein. The mammalian orthologs of DRL and SRC64B, RYK and c-SRC, physically interact as assayed by their co-immunoprecipitation from transfected human 293T cell lysates. The expression of RYK-HA and untagged c-SRC was confirmed by WCE immunoblots. RYK derived from c-SRC-overexpressing cells migrates faster than control RYK species, presumably owing to altered post-translational processing. Endogenous c-SRC protein is visible in lanes 1 and 2 of the lowermost blot.

Fig. 4. SRC64B and DRL and their mammalian orthologs physically associate. (A) Drosophila Kc cells were transfected with the indicated expression constructs, lysates were immunoprecipitated (IP) with antibodies specific to DRL (anti-HA) or SRC64B (anti-MYC) and then immunoblotted (WB) with the reciprocal antibody to detect co-immunoprecipitation. The expression of DRL and SRC64B was confirmed by immunoblotting of the whole-cell extract (WCE). DRL and SRC64B specifically co-immunoprecipitate in the presence or absence of WNT5 protein. (B) The mammalian orthologs of DRL and SRC64B, RYK and c-SRC, physically interact as assayed by their co-immunoprecipitation from transfected human 293T cell lysates. The expression of RYK-HA and untagged c-SRC was confirmed by WCE immunoblot. RYK derived from c-SRC-overexpressing cells migrates faster than control RYK species, presumably owing to altered post-translational processing. Endogenous c-SRC protein is visible in lanes 1 and 2 of the lowermost blot.

We investigated whether this apparent physical interaction between a Drosophila RYK and SFK could also be observed with their mammalian orthologs. HA-tagged mouse RYK and untagged human c-SRC expression constructs were co-transfected into the 293T human embryonic kidney cell line. Cell lysate proteins were immunoprecipitated with anti-c-SRC and immunoblots were probed with anti-HA antibody to visualize RYK. RYK co-immunoprecipitated with c-SRC (Fig. 4B), suggesting they form a complex. RYK precipitated from c-SRC-overexpressing cells migrated slightly faster on SDS-PAGE gels than the RYK-alone control, indicating that some post-translational modification of RYK takes place upon increased expression of c-SRC. Collectively, the results of these co-immunoprecipitation experiments and the axonal localization of both RYK/DRL and SFKs in mammals and Drosophila, make it likely that RYKs and SFKs interact in evolutionarily distant species.

Formation of the DRL-SRC64B complex results in DRL phosphorylation and increased SRC64B activation and requires SRC64B kinase activity

We then evaluated whether the kinase activity of SRC64B was required in the formation or stabilization of the DRL-SRC64B complex. The physical association of DRL and SRC64B was dependent on the kinase activity of SRC64B or an associated tyrosine kinase: treatment of the co-transfected cells with herbimycin A, a tyrosine kinase-specific inhibitor, resulted in their reduced co-immunoprecipitation (Fig. 5A). To further assess the role of SFK kinase activity, we used a mammalian two-hybrid assay in which plasmids expressing a SRC64B and DRL intracellular domain fusion protein were transfected into SFK-deficient cells (Klinghoffer et al., 1999) to eliminate possible interference by the highly conserved endogenous mammalian SFKs. Coexpression of wild-type SRC64B and DRL intracellular domain fusion proteins led to significant increases in luciferase expression above that of the controls, indicating that these proteins physically interact (Fig. 5B). No significant expression of luciferase was observed when catalytically inactive SRC64B (KD) was coexpressed with DRL (Fig. 5B).

The requirement for tyrosine kinase activity in the formation or stability of the SRC64B-DRL complex raised the question as to whether either DRL or SRC64B displayed increased tyrosine phosphorylation upon coexpression. Evaluation of tyrosine phosphorylation of whole-cell extract proteins derived from cells transiently transfected with DRL, SRC64B, or both expression constructs revealed a dramatic increase in the phosphorylation of a 75 kDa protein(s) in the doubly transfected cells (Fig. 6A). The tagged DRL and SRC64B proteins both displayed apparent molecular weights of ~75 kDa on denaturing gels. Therefore, to investigate whether this species includes DRL, we initially immunoprecipitated the DRL-containing complex with anti-DRL, dissociated it by boiling and immunoprecipitated DRL with anti-HA (DRL). Anti-phosphotyrosine immunoblots revealed that DRL tyrosine phosphorylation is increased upon its coexpression with SRC64B (Fig. 6B).

SFKs are known to be differentially phosphorylated at specific tyrosine residues depending on their state of activation (reviewed by Roskoski, 2005). We therefore evaluated the degree of phosphorylation of the SRC64B tyrosine at position 434, which is phosphorylated in catalytically active SRC64B (O’Reilly et al., 2006). Anti-PY434SRC64B immunoblot analysis of whole-cell lysates from cells transiently transfected with DRL, SRC64B, or both expression constructs revealed a significant increase in the phosphorylation of a 75 kDa protein(s) in the doubly transfected cells (Fig. 6A). The tagged DRL and SRC64B proteins both displayed apparent molecular weights of ~75 kDa on denaturing gels. Therefore, to investigate whether this species includes DRL, we initially immunoprecipitated the DRL-containing complex with anti-DRL, dissociated it by boiling and immunoprecipitated DRL with anti-HA (DRL). Anti-phosphotyrosine immunoblots revealed that DRL tyrosine phosphorylation is increased upon its coexpression with SRC64B (Fig. 6B).
transfected with SRC64B and DRL expression plasmids and immunoblotted with anti-PY434SRC64B revealed that at least some of the SRC64B protein bound to DRL is catalytically active (Fig. 6D).

**WNT5/DRL-mediated signaling does not affect the TCF/LEF canonical Wnt target**

As a previous study indicated that engagement of mammalian RYK by Wnt proteins results in the transduction of a canonical Wnt signal, culminating in increased TCF/LEF-dependent transcription (Lu et al., 2004), we evaluated whether or not their Drosophila orthologs also signal through this pathway. Drosophila S2 tissue culture cells were transfected with the indicated combinations of expression plasmids and a TCF/LEF-responsive luciferase reporter gene [Super8XTopFlash (Veeman et al., 2003) or a control reporter (Super8XFopFlash)] and luciferase levels were measured 48 hours post-transfection. Transfection of a construct encoding a canonical Wnt protein, Wingless (WG), resulted in high TCF/LEF-dependent luciferase expression. S2 cells express both fz and fz2 as assayed by quantitative RT-PCR (data not shown), precluding the necessity of transfecting expression plasmids for either of the canonical Fz receptors. Transfection of a WNT5-expressing plasmid alone or in combination with a DRL-expressing plasmid did not result in expression of luciferase above control levels (Fig. 7), suggesting that Wnt5/drl-mediated signaling does not increase TCF/LEF activity.

As the mammalian WNT5A protein acting through the ROR receptor has been shown to inhibit canonical Wnt signaling (Mikels and Nusse, 2006), we evaluated whether or not WNT5 interaction with DRL might similarly block canonical signaling. Coexpression of WG, WNT5 and DRL resulted in luciferase expression levels similar to those seen with WG alone (Fig. 7), suggesting that Wnt5/drl-mediated interactions do not apparently inhibit contemporaneous canonical Wnt signaling.

**DISCUSSION**

The genetic data presented in this report indicate that the Drosophila SRC64B and SRC42A SFKs play redundant roles in establishing the embryonic CNS commissures. Furthermore, we present evidence that SRC64B, and possibly SRC42A, act in commissure formation, as members of the WNT5/DRL signaling pathway. The other SFK, SRC42A, which is also expressed in the embryonic CNS (Takahashi et al., 2005), plays partially redundant roles to those seen with WG alone (Fig. 7), suggesting that Wnt5/drl-mediated interactions do not apparently inhibit contemporaneous canonical Wnt signaling.
of SRC64B in other tissues (Takahashi et al., 2005; Tateno et al., 2000; Harris and Beckendorf, 2007). Heterozygosity for Src42A did not suppress the Wnt5 midline glial expression phenotype, possibly because SRC42A is not limiting under these conditions.

Third, we find that reduction of Src64B expression levels in the Sema2B+ AC-crossing neurons by transgenic RNA interference results in axon pathfinding phenotypes similar to those seen in Wnt5 (Fradkin et al., 2004) and drl (Yoshikawa et al., 2003) mutants. The aberrant pathfinding of Src64B dsRNA-expressing Sema2b+ neurons, which normally project through the AC, indicates that wild-type expression levels of SRC64B are required for their correct routing. The incomplete penetrance of this phenotype possibly reflects the presence of wild-type levels of SRC42A, which we have shown is at least partially redundant with SRC64B. Alternatively, expression of Src64B-targeted dsRNA might not adequately reduce SRC64B expression levels to effect full penetrance.

Fourth, we demonstrate that drl and Src64B/Src42A interact synergistically in an axon-switching assay. Increased SFK neuronal expression levels alone could not force axons that normally traverse the PC to cross in the AC. Use of a DRL-expressing transgene facilitating only moderate switching showed that elevated Src64B or Src42A expression levels significantly increased switching. SFK catalytic activity is required to enhance DRL-dependent switching, as kinase activity-deficient SRC64B did not increase switching. Thus, catalytically active SFKs can synergize with limiting levels of DRL to induce commissure switching, and presumably act to control the wild-type trajectories of the AC axons. Furthermore, Src64B and drl have previously been reported to interact genetically during pupal brain development (Nicolai et al., 2003) and it was also recently reported that drl genetically interacts with the SFK genes to control salivary gland migration (Harris and Beckendorf, 2007).

Fig. 6. DRL is tyrosine phosphorylated in a SRC64B-dependent manner and DRL coexpression activates SRC64B. (A) DRL and SRC64B co-transfected cell lysates contain a predominant protein species with increased tyrosine phosphorylation (asterisk). Drosophila S2 cells were transfected with the indicated plasmids and WCEs analyzed by immunoblotting with an anti-phosphotyrosine mAb. (B) DRL is phosphorylated in a SRC64B-dependent manner. Lysates of S2 cells transiently transfected with the indicated constructs were immunoprecipitated with anti-DRL antiserum, complexes washed, disrupted by boiling and DRL reprecipitated with anti-HA antiserum and analyzed by anti-phosphotyrosine immunoblotting. V, vector alone. (C) Coexpression of DRL and SRC64B results in a WIF and cytoplasmic domain-dependent activation of SRC64B. S2 cells were transfected as follows: lane 1, SRC64B[WT] only; lane 2, DRL[WT] + SRC64B[WT]; lane 3, DRL[ΔCyto] + SRC64B[WT]; lane 4, DRL[ΔWIF] + SRC64B[WT]; lane 5, DRL[WT] + SRC64B[KD]; lane 6, DRL[ΔTBC] + SRC64B[WT]; and lane 7, DRL[ΔPDZ] + SRC64B[WT]. WCEs were immunoblotted to detect active SRC64B (anti-PY434SRC64B), pan-SRC64B (anti-MYC) and DRL (anti-HA). All transfections contained SRC64B-MYC except lane 5. Quantitation of a similar experiment performed in triplicate is shown in Fig. S5 in the supplementary material. (D) DRL-associated SRC64B is, at least in part, catalytically active. Lysates from cells transfected as indicated were immunoprecipitated with anti-DRL and analyzed by anti-PY434SRC64B immunoblotting. Control anti-MYC (SRC64B) and anti-HA (DRL) blots are shown.
SFK requirement in Wnt/RYK signaling

Supporting these previous observations and the genetic data presented here, we found that SRC64B and DRL physically interact, as assayed by co-immunoprecipitation. The formation or stability of this complex is apparently dependent upon SFK kinase activity as shown by the failure of the proteins to co-immunoprecipitate from lysates derived from cells treated with herbimycin A, a tyrosine kinase inhibitor. Further support for this involvement of SFK in the assays employed here. Such changes might lead to alterations in tyrosine kinase target specificity, such as those demonstrated for the Src-interacting Na+/K+-ATPase (Tian et al., 2005).

Fig. 7. The expression of WNT5 and DRL neither activates nor represses canonical TCF/LEF-dependent transcription. Drosophila S2 cells were transfected in triplicate with the expression plasmids indicated and either the TCF/LEF-dependent transcription reporter Super8XTopFlash (black bars) or the control Super8XTopFlash (white bars). Luciferase expression levels were determined, normalized to internal Renilla controls and plotted.

Binding of Wnt protein to RYK stimulates TCF/LEF-dependent transcription via the Dishevelled adaptor protein in transfected cells, suggesting that the RYK pathway overlaps with the canonical Wnt pathway (Lu et al., 2004). Our data indicate, however, that the Drosophila WNT5/DRL signaling pathway does not regulate TCF/LEF-dependent transcription. Transfection of Drosophila S2 tissue culture cells, which respond to the canonical Wg ligand, with DRL and WNT5 expression constructs does not increase TCF/LEF-dependent reporter gene expression. Furthermore, unlike the recently reported WNT5A-ROR interaction (Mikels and Nusse, 2006), Wnt5/drl-mediated signaling does not apparently block contemporaneous canonical Wnt signaling. The Drosophila WNT5 protein, however, can also signal via Fz family receptors to activate a non-canonical Wnt pathway (Srahna et al., 2006). Although our data render it unlikely that SRC64B is a member of the canonical Wnt signaling pathway, it might act in a pathway parallel to canonical Wnt signaling as has been reported for SFKs during convergent extension cell movement in zebrafish (Jopling and den Hertog, 2005) and during cell fate specification and cleavage orientation in C. elegans (Beil et al., 2002).

It is presently unclear whether the SFKs relay a WNT5/DRL signal, and if so, to what downstream pathway members and by what mechanisms. Our observations that increased Src64B and Src42A expression levels enhance drl-mediated commissure switching of axons in a sensitized background and that the SFKs and DRL physically interact, suggest the possibility that DRL dictates the target specificities of the bound SFKs by co-localizing them with potential targets. This hypothesis is attractive because the SFKs are widely expressed throughout the ventral nerve cord and are also likely to act downstream of other axonal receptors, the mammalian orthologs of which [e.g. the TRKB (NTRK2), ephrin A and netrin receptors] are known to interact with the SFKs (Iwashaki et al., 1998; Knoll and Drescher, 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Furthermore, DRL-dependent asymmetric localization or regulation of SFKs within the growth cone might mediate axon steering. Such localized changes in growth cone SFK activity effect axon turning in cultured Xenopus primary neurons (Robles et al., 2005).
Although the identification of the relevant SFK targets and of other members of the WNT5/DRL-mediated signaling pathway lies ahead, the data presented here indicate that the catalytically active SFKs are required for WNT5-mediated axon repulsion via the catalytically inactive DRL receptor. Identification of other pathway members, including potential SFK targets, through a combination of genetic and biochemical approaches should further reveal the mechanisms by which Wnt proteins signal through the RYKs.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/13/3277/DC1

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