Regulation of cell–cell contacts in developing *Drosophila* eyes by *Dsrc41*, a new, close relative of vertebrate *c-src*

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In *Drosophila*, *Dsrc64* is considered a unique ortholog of the vertebrate *c-src*; however, we show evidence to the contrary. The closest relative of vertebrate *c-src* so far found in *Drosophila* is not *Dsrc64*, but *Dsrc41*, a gene identified for the first time here. In contrast to *Dsrc64*, overexpression of wild-type *Dsrc41* caused little or no appreciable phenotypic change in *Drosophila*. Both gain-of-function and dominant-negative mutations of *Dsrc41* caused the formation of supernumerary R7-type neurons, suppressible by one-dose reduction of *boss*, *sev*, *RasI*, or other genes involved in the Sev pathway. Dominant-negative mutant phenotypes were suppressed and enhanced, respectively, by increasing and decreasing the copy number of wild-type *Dsrc41*. Colocalization of *Dsrc41* protein, actin fibers and *DE-cadherin*, and *Dsrc41*-dependent disorganization of actin fibers and putative adherens junctions in precluster cells suggested that *Dsrc41* may be involved in the regulation of cytoskeleton organization and cell–cell contacts in developing ommatidia.

[Key Words: Protein tyrosine kinase; *Drosophila*; *src; Dsrc41; cadherin; boss]

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The Src tyrosine kinase family in vertebrates consists of nine members [for review, see Cooper and Howell 1993], at least three of which—*src*, *yes*, and *fyn*—are widely expressed in a variety of cells including those in the neural ectoderm and developing cerebellum (Cotton and Brugge 1983; Fults et al. 1985; Manness et al. 1986; Zhao et al. 1990). These tyrosine kinases may participate in developmental processes such as neural and hematopoietic cell differentiation (Molina et al. 1992; Yagi et al. 1993). Studies using knockout mice suggest that compensatory interactions occur between Src family tyrosine kinases. Only a restricted phenotype, or no overt dysfunctional consequences is observed in a loss-of-function mutant of *src*, *yes*, and *fyn*, whereas most *src*/*fyn* or *src*/*yes* double mutants die perinatally and a considerable number of *fyn*/*yes* double mutants undergo degenerative renal damage with consequent diffuse segmental glomerulosclerosis (Stein et al. 1994).

Elimination of the conserved tyrosine residue in the carboxyl terminus endows Src family tyrosine kinases with transforming activity. This activity is thought to be induced by derepression of tyrosine kinase activity (Cartwright et al. 1987), which is normally repressed via stoichiometric phosphorylation of the carboxy-terminal tyrosine residue by tyrosine kinase kinases such as Csk. Individuals with homozygous deficiency of *csk* are embryonic lethal (Nada et al. 1993; Imamoto and Soriano 1993). Because of this repression mechanism, the overexpression of *c-src* in fibroblasts has no significant effect on cell growth or morphology, nor overall abundance of tyrosine-phosphorylated proteins (Shalloway et al. 1984; Iba et al. 1985).

In *Drosophila*, three *src*-related genes, *Dash* (Henkemeyer et al. 1988), *Dsrc29* (Gregory et al. 1987), and *Dsrc64* (Simon et al. 1985), have been identified using a *v-src* fragment as a probe. Only the polypeptide encoded by *Dsrc64* (*Dsrc64*) resembles, in its entire structure, vertebrate Src and is presumed to belong to the Src family (Simon et al. 1985). *Dash* may be a counterpart of vertebrate abl (Henkemeyer et al. 1988), whereas *Dsrc29* is suggested to be in the same family as mammalian tyrosine kinase genes *itk* and *tec* (Tsukada et al. 1993). Recently, however, Kussick et al. (1993) showed *Dsrc64* to have properties considerably different from those of vertebrate c-Src. In contrast to vertebrate c-Src overexpressed in fibroblasts (Iba et al. 1985), wild-type *Dsrc64* overexpressed in *Drosophila* was phosphorylated not only at the carboxy-terminal tyrosine residue but at major in vitro autophosphorylation sites as well (Kussick and Cooper 1992), causing embryonic lethality or abnormal differentiation (Kussick et al. 1993). Thus possibly,
carboxy-terminal tyrosine phosphorylation of Dsrc64 is substoichiometric (Kussick and Cooper 1992). Here, we show isolation and characterization of Dsrc41, a new src-related gene in Drosophila. Nucleotide sequence analysis and analysis of phenotypes of gain-of-function and dominant-negative mutations in developing retina and embryos suggested that Dsrc41 is the closest relative of vertebrate c-src found so far in Drosophila and may play an essential role in cytoskeleton organization and cell–cell contacts in development.

Results

Molecular cloning of Dsrc41, the closest relative of vertebrate c-src found so far in Drosophila

dtk-5 is a PCR fragment encoding a partial tyrosine kinase sequence (Shishido et al. 1991). During the course of searching dtk5-related genes in Drosophila, we discovered two λ clones, λgfk1 and λgfk2 [Fig. 1A], which together encode the putative polypeptide showing much greater similarity in amino acid sequence to vertebrate c-Src than Dsrc64 [see Fig. 2B]. As its cDNA hybridized at 41B or 41C on the second chromosome (data not shown), we refer to the gene hereafter as Dsrc41.

Nucleotide sequences of the longest cDNA and relevant genomic DNA fragments were determined [Fig. 2A]. The longest cDNA was 2.9 kb in length, which would agree well with the 3-kb RNA observed in Northern blots [Fig. 1B]. Dsrc41 contains 9 exons that would code for a polypeptide of 517 amino acids residues [Figs. 1A and 2A]. The Dsrc41 gene product [Dsrc41] resembled the vertebrate Src family kinase consensus over the entire SH1 (catalytic), SH2, and SH3 domains, the amino-terminal myristylation site, and the carboxy-terminal tail [Fig. 2A,B].

As shown in Figure 2B, the kinase domain of Dsrc41 was much more similar in amino acid sequence to those of vertebrate Src members than Dsrc64. On average 173 amino acids were conserved between Dsrc41 and five vertebrate Src members [Src, Yes, Yrk, Fgr, and Fyn], whereas only 148 amino acids were invariant between Dsrc41 and Dsrc64. Similarity in amino acid sequence between Dsrc41 and vertebrate Src members was also evident in SH3 and the carboxy-terminal tail [Fig. 2B], making possible unequivocal assignment of Tyr-511 of Dsrc41 to c-Src Tyr-527, whose phosphorylation is essential for the regulation of kinase activity within cells (Cartwright et al. 1987). Structural resemblance between vertebrate c-src and Dsrc41 can be extended further to exon–exon junctions [Fig. 2E]. In contrast, no correlation in exon–exon junctions was detected between Dsrc64 and c-src. Dsrc64 thus likely forms its own group distantly related to Dsrc41 and vertebrate src family members [Fig. 2C].

Drosophila may contain no close relative of Dsrc41, because as shown in Figure 1C, no band other than those for Dsrc41 could be detected by hybridization under less stringent conditions.

Temporal and spatial expression of Dsrc41 RNA

Poly[A]+ RNA extracted from embryos, larvae, pupae, and adults was examined by Northern blotting [Fig. 1B]. A single transcript, 3 kb long, was found in both embryos and pupae. Its spatial patterns in embryos and imaginal discs were also examined, as shown in Figure 3. In very early embryos [A], little or no Dsrc41 RNA was detected, suggesting the level of its maternal expression to be quite low, if any. Dsrc41 RNA was first discernible at stages 2 or 3 (Campos-Ortega and Hartenstein 1985). At...
**Figure 2.** (See facing page for legend.)
the cellular blastoderm stage, it was expressed weakly in virtually all cells (C). Uniform but somewhat stronger expression persisted until the end of germ-band retraction (D). During stages 13–16, relatively high expression was recognized in visceral mesoderm (E), hindgut, brain, anal pads, and ventral ganglia (F,G). No signal of Dsrc41 RNA was detected in embryos homozygous for a deficiency of Dsrc41 (H). In third instar larvae, Dsrc41 RNA expression was still strong in the central nervous system (CNS) (M). Almost all cells expressed Dsrc41 RNA in wing (L), leg (N), and eye–antennal (O,P) discs. In contrast, Dsrc64 expression in eye discs has been reported to be restricted to photoreceptor precursors posterior to the morphogenetic furrow (Kussick et al. 1993).

In vivo functions of Dsrc41 and vertebrate c-Src are similarly regulated by the carboxy-terminal conserved tyrosine residue

Overexpressed c-src in fibroblasts induces no appreciable phenotypic change (Shalloway et al. 1984), whereas elimination of the carboxy-terminal tyrosine residue results in activation of intrinsic catalytic activity and malignant transformation (Cartwright et al. 1987). Using Drosophila compound eye and embryonic systems, we examined effects of the overexpression of Dsrc41 with or without the conserved carboxy-terminal tyrosine residue.

DNA fragments containing either wild-type Dsrc41
a constitutively active mutant containing a Tyr → Phe substitution at position 511 \(D_{sr} c_{41}^T\) were subcloned into \(P\)-based expression vectors possessing one or two \(sevenless\) \(sev\) enhancers \(se\) or \(se^2\) associated with the \(hsp70\) promoter [Fig. 2D]; \(se^2\) is more active than \(se\) [Bowtell et al. 1991]. Thus, the expression of the inserted \(D_{sr} c_{41}\) gene is bipartite; the \(hsp70\) promoter enables heat shock-inducible, ubiquitous expression, whereas \(sev\) enhancers drive high expression in a limited species of ommatidial cells. Using these constructs, three and two transformant lines containing \(se^2-D_{sr} c_{41}^T\) [WT-A, WT-B, and WT-C] and \(se-D_{sr} c_{41}^T\) \(sev\) [YF-A and YF-B], respectively, were obtained. In situ hybridization showed these transgenes to be overexpressed in the region posterior to the morphogenetic furrow in eye discs and heat-shocked embryos [Fig. 3].

As with flies with activated Sev tyrosine kinase \(Sev^{SI}\) [Basler et al. 1991], \(D_{sr} c_{41}^T\) flies exhibited rough eyes [Fig. 4B] associated with extra R7-type neurons resistant to the \(outer\ rhabdomeres\ absent\) \(ora\) mutation [Stark and Sapp 1987] [Fig. 5B,E]. An ommatidium heterozygous for the \(se-D_{sr} c_{41}^T\) transgene insertion [YF-A] contained two to three R7-type neurons [Table 1], and the value increased with increasing copy of the inserted transgene [data not shown]. In contrast, no appreciable morphological change in eye structure was detected in any of three transgenic lines with \(se^2-D_{sr} c_{41}^T\) [Fig. 4C]. This finding is important, as under similar conditions, flies with a single copy of the wild-type \(D_{sr} c_{64}\) transgene showed extensively deformed eye morphology [Kussick et al. 1993].

\(D_{sr} c_{41}^T\) and \(D_{sr} c_{41}^T\) transgenes were overexpressed in embryos by heat shock at 37°C for 30 min. Overexpressed \(D_{sr} c_{41}^T\) gave no appreciable effect on viability, whereas the overexpression of \(D_{sr} c_{41}^T\) was deleterious.

Figure 5. Tangential sections at the R7 level. [A] Wild type. [B] A transformant line [YF-A] heterozygous for the \(se-D_{sr} c_{41}^T\) insertion. [C] A transformant [WT-A] homozygous for the \(se^2-D_{sr} c_{41}^T\) insertion. [D] Outer photoreceptor rhabdomeres are absent from the \(ora\) mutant. [E] Supernumeral rhabdomeres in YF-B are stable against the \(ora\) background. [F–H] Fly strains doubly heterozygous for \(se-D_{sr} c_{41}^T\) insertion [YF-A] and one of a mutation of \(sevenless\) pathway components. [F] \(Ras1^{ep}\), [G] \(boss\). [H] \(sev^{D2}\). [I] Heterozygote for \(se^2-D_{sr} c_{41}^{K\bar{R}}\) insertion [KR-A]. [J,K] \(se^2-D_{sr} c_{41}^{K\bar{R}}\) heterozygous for \(se^2-D_{sr} c_{41}^T\) insertions [I] or \(Ras1^{ep}\) [K]. [L] \(se^2-D_{sr} c_{41}^{K\bar{R}}\) heterozygous for \(sev^{D2}\). [M] Double heterozygote for \(se^2-D_{sr} c_{41}^{K\bar{R}}\) insertion and a \(D_{sr} c_{41}\) deficiency. [N] Heterozygote for \(se^2-D_{sr} c_{41}^{K\bar{R}}\) insertion [KR-YF-A].

\[D_{sr} c_{41}, a\ new\ Drosophila\ src\ gene\]
Table 1. sE-Dsrc41YF phenotype is affected by mutations in the sev signaling pathway

| Genotype       | Average number of R7-type neurons | Number of ommatidia |
|----------------|----------------------------------|---------------------|
| Wild type      | 1.0 ± 0.0 (±0.0)                 | 234                 |
| WT-A           | 1.0 ± 0.0 (±0.1)                 | 185                 |
| YF-A           | 2.7 ± 0.1 (±1.2)                 | 362                 |
| dsrc41; YF-A   | 1.0 ± 0.0 (±0.2)                 | 250                 |
| YF-A; sev1, YF-A | 1.0 ± 0.0 (±0.0)               | 200                 |
| YF-A; sev1, YF-A | 1.1 ± 0.0 (±0.4)                | 398                 |
| YF-A; sev1, YF-A | 1.1 ± 0.1 (±0.4)                | 387                 |
| YF-A; sev1, YF-A | 1.3 ± 0.1 (±0.5)                | 267                 |
| YF-A; sev1, YF-A | 1.6 ± 0.1 (±0.8)                | 228                 |
| YF-A; sev1, YF-A | 1.9 ± 0.1 (±0.8)                | 340                 |
| YF-B           | 2.1 ± 0.1 (±0.9)                 | 350                 |
| YF-B; sev1/boss' | 0.6 ± 0.0 (±0.7)                | 367                 |
| sev1, YF-A     | 1.4 ± 0.1 (±0.6)                 | 309                 |
| YF-A; sev1, YF-A | 1.4 ± 0.1 (±0.6)                | 309                 |
| YF-A; sev1, YF-A | 2.4 ± 0.2 (±0.8)                | 284                 |
| YF-A; sev1, YF-A | 2.6 ± 0.1 (±0.8)                | 200                 |

For YF-A series, the transgene (sE-Dsrc41YF) is heterozygous, whereas it is hemizygous in the case of YF-B. Two kinds of deviations are shown: The first one was calculated using the total number of eyes examined, and the second one, that of ommatidia. In the third column, only the total number of ommatidia examined is shown.

Close inspection of embryos homozygous for Dsrc41YF heat-shocked at 4–7 hr of development, and cultured at 25°C until 13–16 hr of development demonstrated that the overexpression of the activated Dsrc41 results in the failure of CNS shortening and dorsal closure, leading to embryonic lethality [Fig. 3,J,K]. In contrast, the overexpression of wild-type Dsrc41, along with that of its activated version, has been shown to be embryonic lethal [Kussick et al. 1993]. It may thus follow that like vertebrate c-Src, Dsrc41 activity is repressed by stoichiometric phosphorylation of the carboxy-terminal conserved tyrosine residue [Tyr-511].

Wild-type Dsrc41 is essential for normal eye morphogenesis

c-Src with an inactive ATP-binding site because of a Lys→Arg substitution has been shown to be capable of serving as an effective antagonist of wild-type c-Src and, hence, to exhibit Src kinase-negative phenotypes [Chang et al. 1995]. Thus, by P-mediated transformation [Fig. 2D], four transgenic fly lines [KR-A, KR-B, KR-C, and KR-D] were made that expressed a dominant-negative version of Dsrc41 [Dsrc41KR] driven by the sE enhancer.

All four transgenic fly lines exhibited similar rough eye phenotypes [Fig. 4D]. Cobalt sulfide staining [Fig. 6C] showed the shape and distribution of preclusters to be considerably affected. Either R3 or R4 precursors, or both, appeared to stain more weakly than their wild-type counterparts. As shown below, rhodamine-phalloidin and anti-DE-cadherin staining suggested that putative adherens junctions situated between R3 and R4 precursors is disorganized extensively in Dsrc41KR flies.

H214 is an enhancer-trap marker used to identify precursors for extra R7-type neurons [Mlodzik et al. 1992]. The ectopic expression of H214 first became discernible in a very small fraction of late Dsrc41KR larvae [rows 17 and more posterior, Fig. 6H], and ommatidia having extra H214-positive cells increased in number extensively during pupal stages [Fig. 6L]. This suggests that the formation of ectopic pro-R7s in Dsrc41KR flies initiates at a stage much later than that for the formation of authentic R7 precursors.

During pupal stages, stacking of nuclei of two seven-up [svp]-positive cells became more apparent, and loss of svp-positive cells was observed in a small fraction of pupal ommatidia [Fig. 6J]. In Dsrc41KR adult eyes, rhabdomere patterns and ommatidial spacing were highly abnormal. Most ommatidia contained one or two additional R7-type neurons, whereas one or a few outer photoreceptors were absent from 50% ommatidia [Figs. 5I and 7J]. Taken together, these results indicate that the dominant-negative form of Dsrc41 affects many steps of eye morphogenesis.

If, as expected, Dsrc41KR serves as an antagonist of wild-type Dsrc41, eye phenotypes induced by the Dsrc41KR transgene should be suppressed and enhanced, respectively, by the increment and decrement of the dose of wild-type Dsrc41. Figures 4, E and F, 5, J and M, 6, B and D, and 7 show this to be the case. All Dsrc41KR-dependent phenotypes examined so far were suppressed and enhanced, respectively, by simultaneous expression of an additional copy of a transgene, sev'–Dsrc41+ and one-copy reduction of the endogenous Dsrc41 [Figs. 4E,F, 5J,M, and 6B,D]. Note that the additional introduction of a heterozygous deficiency of the Dsrc41 locus results in the absence of R3/R4 photoreceptors stained strongly by cobalt sulfide [Fig. 6D].

The Dsrc41KR transgene might function only to titrate out a negative regulator [such as Csk] to increase the fraction of the active, unphosphorylated form of endogenous [wild-type] Dsrc41. Activated Dsrc41 thus generated might undergo relocation to bind to target molecules [Kaplan et al. 1994] to consequently induce gain-of-function phenotypes similar to those observed in Dsrc41YF flies. But this appeared unlikely, as [1] timing of the appearance of Dsrc41KR-dependent defects differed considerably from that of Dsrc41YF-dependent defects, and [2] Dsrc41KR phenotypes were considerably enhanced by an additional YF mutation that may neutralize kinase activation by stimulating competitive inhibition upon target binding [Fig. 2D; see Fig. 5I,N]. Dsrc41 may thus be essential for normal eye morphogenesis.

boss-dependent, extra R7 formation in Dsrc41YF ommatidia

R7 formation in the wild type depends on Sev kinase activated by Boss expressed on the surface of R8 [Kramer et al. 1991]. Although R7 and cone cell precursors express Sev, only pro-R7s are capable of coming into con-
Dsrc41, a new Drosophila src gene interacts with R8 and receiving Boss signals [Tomlinson et al. 1987]. This R7–R8 interaction may be demonstrated by genetic analysis along with Boss internalization [Reinke and Zipursky 1988; Kramer et al. 1991].

Table 1 and Figure 5, G and H, show that the number of extra R7-type neurons in Dsrc41^{Ty} flies decreases considerably with the one-dose reduction of boss or sev. This is of interest, as no effect of boss on gain-of-function mutations of Sev pathway genes has been reported [Basler et al. 1991; Fortini et al. 1992]. Also, as in the case of the authentic pro-R7 [Fig. 6E], Boss was found to be internalized in a small but appreciable fraction of Dsrc41^{Ty} ommatidial cells [Fig. 6F], which are most likely to be precursors for extra R7-type neurons. Our preliminary experiment also indicated a one-dose reduction of Dsrc41 to give no appreciable effect on Sev^{311} (data not shown), suggesting that Dsrc41 functions upstream of Sev. sev–Dsrc41^{Ty} would thus appear to endow cone cell precursors with the ability to make contact with R8 so as to receive the Boss signals for neuralization.

Requirement of the Ras1/MAPK signal transduction pathway beginning with Boss for both gain-of-function and dominant-negative phenotypes of Dsrc41

Genetic analysis using haploinsufficiency showed that the entire Ras1/mitogen-activated protein kinase (MAPK) signal transduction pathway is essential for Dsrc41^{Ty}-dependent formation of R7-type neurons [Table 1]; one-copy reduction of endogenous drk, Sos, or...
Rasl virtually completely suppresses ectopic neuralization. Enhanced effects of these elements may be partly attributable to the fact that not only the Boss/Sev signal but also the DER [Drosophila epidermal growth factor (EGF) receptor] signal contributes to neuralization in the Dsrc41YF mutant (Table 1). However, unlike the Sev pathway in the authentic R7 (Simon et al. 1991), cdc37 and E[sev]3D may not be required for ectopic neuralization in the Dsrc41YF mutant.

Dominant-negative phenotypes of Dsrc41 also depend on the Ras1/MAPK signal transduction pathway beginning with Boss (Figs. 5 and 7). Lost outer photoreceptors were virtually completely recovered by one-copy reduction of either Ras1 or boss or the absence of sev. The formation of extra R7-type neurons was also partially reversed (Figs. 5 and 7). However, it should be noted here that the Boss protein expressed in R8 may not be related to these phenotypes because neuralization of extra R7-like cells and loss of outer photoreceptors in Dsrc41KR flies occur mainly during pupal stages when Boss expression is not specific to R8 but rather ubiquitous and non-functional (Kramer et al. 1991).

Localization of Dsrc41YF-LacZ in the close vicinity of DE-cadherin-positive, putative adherens junctions

In vertebrate cultured cells, Src is relocalized to focal adhesions following activation and controls cytoskeleton organization and cell signaling (Kaplan et al. 1994). Although no focal adhesions have been recognized in larval eyes, larval ommatidial cells are connected by adherens junctions (Longley and Ready 1995), which are presumably functional analogs of focal adhesions in the cell–cell adhesion system (Gumbiner 1996). In vertebrates, classic cadherins such as E-cadherin have been shown to be essential not only for formation of adherens junctions but also for the maintenance of cell–cell contacts and actin cytoskeleton (Takeichi 1991; Gumbiner 1996). Thus, we first examined the spatial distribution of DE-cadherin, a putative ortholog in Drosophila of vertebrate E-cadherin (Oda et al. 1994), and compared it with that of the Dsrc41YF–LacZ fusion protein.

Figure 6M shows the distribution of DE-cadherin signals obtained by confocal microscopy, indicating that DE-cadherin distributes widely along boundaries of many ommatidial and nonommatidial cells. Because the distribution of actin fibers (cytoskeleton) visualized by rhodamine-phalloidin was very similar, if not identical, to that of DE-cadherin (Fig. 6T), DE-cadherin-positive regions found in larval eyes would appear to correspond to adherens junctions and/or their close relatives in developing eyes.

In normal eye development, ommatidial formation is
lineage independent, and each ommatidial precursor is assembled through the stepwise recruitment of cells into stereotyped clusters [for review, see Wolff and Ready 1993]. Developing photoreceptors express neural antigen in a fixed sequence: R8 expresses neural antigens first and is followed by the R2/R5 pair and then by the R3/R4 pair. Interestingly, as shown in Figure 6S, the order and timing of the appearance of strong cadherin signals were virtually identical to those of neural antigens (for neural antigen expression, see Wolff and Ready 1993). Strong cadherin signals first appeared along boundaries of R8 in row 2. Photoreceptors R2 and R5 begin expressing strong cadherin signals in rows 3 and 4. Strong cadherin signals appeared along R3–R4 boundaries in row 5. It may thus follow that the formation of adherens junctions is initiated at a very early stage of photoreceptor development.

In row 9 or 10, three distinct types of DE-cadherin signals were recognized. Type I signals distributed along the apical-most boundaries of cells not yet recruited into ommatidia and putative cone cell precursors (Fig. 6M). Type II signals (0.5–2 μm beneath the apical surface) were found along boundaries between cone cells and photoreceptors (Fig. 6N). Type III signals, ~1 μm in height and situated at ~1.5 μm beneath the apical surface, were readily apparent along boundaries between neighboring photoreceptor precursors (Fig. 6N). Locations of type II and type III signals appeared to coincide with those of zonula adherens visualized by electron microscopy [Longley and Ready 1995], suggesting that type II and type III signals may correspond to zonula adherens.

Recently, this notion was confirmed further in an independent experiment (T. Uemura and H. Oda, pers. comm.).

The distribution of the fusion protein consisting of activated Dsrc41 and LacZ [sE2–Dsrc41Y7lacZ, Fig. 2D] was examined using anti-LacZ (or Escherichia coli β-galactosidase) antibody. sE2–Dsrc41Y7-lacZ flies exhibited eyes slightly rough in appearance. Confocal images of anti-LacZ and anti-DE-cadherin antibody double staining gave strong indication that Dsrc41–LacZ signals considerably overlap three types of DE-cadherin signals (Fig. 6R). However, it should be noted that type II signals of Dsrc41–LacZ exhibit marked apical extension (Fig. 6O), and the appearance of type III signals of DE-cadherin precedes those of Dsrc41–LacZ (Fig. 6, cf. Q and R). Taken together, these results suggest that activated Dsrc41 is localized in close proximity to DE-cadherin-positive adherens junctions and/or other types of DE-cadherin aggregates.

Failure in formation of stable putative adherens junctions between adjacent precluster cells expressing Dsrc41KR

As shown in Figure 6C, a transient expression of Dsrc41KR in precursors for R3 and R4 resulted in ommatidial disorders in preclusters. To examine whether these defects are related to possible alterations in cytoskeleton organization and/or cell–cell contacts, the distribution of DE-cadherin and actin fibers in Dsrc41KR preclusters was examined. As shown in Figure 6, U and W, little or no signal for actin fibers and DE-cadherin could be detected along boundaries between R3 and R4 precursors. DE-cadherin and actin fibers signals along R3/R2 and R4/R5 boundaries decreased considerably in intensity. Therefore, it may follow that the reduction of Dsrc41 kinase activity results in disorganization of cytoskeletal actin fibers and putative adherens junctions containing DE-cadherin, which is presumed to be responsible for cell–cell contacts [Gumbiner 1996].
A part of the Ras1/MAPK signal may originate from Dsrc41 itself

Table 1 shows not only Sev but also DER to be involved in Dsrc41^{1/2}-dependent neuralization. Vertebrate EGF receptor tyrosine kinase has been shown to be activated via association with v-Src [Maa et al. 1995]. Therefore, similarly to the vertebrate EGF receptor, might be activated by Dsrc41^{1/2}, the activated version of Dsrc41. In vertebrate cultured cells, v-Src, or the activated version of Src, is localized to focal adhesion contacts to phosphorylate FAK and/or p62, whose phosphorylation provides binding sites for Grb2 [Drtk] and/or Sos [Richard et al. 1995]. Our results indicate (activated) Dsrc41 to be localized in putative adherens junctions along with more apical regions. Thus, it is also possible that a part of the Ras signal for neuralization may originate from the activated Dsrc41 itself.

Materials and methods

Plasmids

pYCl.8, a P vector with a vermillion marker, was obtained from L. Searles [Fridell and Searles 1991]. P plasmids expressing Dsrc41 with or without mutations were made as follows. The hsp70 promoter and polyadenylation signal, respectively, were introduced into the XhoI and HpaI–NotI sites of pYCl.8M, a derivative of pYCl.8 with multilocation sites [F. Takahashi, unpubl.] to generate pVH9. Then, a 1.8-kb Scal–Kpnl fragment of Dsrc41 cDNA with or without mutations [see below] was introduced into the BgIII–HpaI site of pVH9. Finally, one or two copies of a 670-bp sev enhancer [Bowtell et al. 1991] were introduced between the SalI and SmaI sites just upstream of the Dsrc41 minigene. se–Dsrc41^{1/2}–lacZ was made similarly. The lacZ fragment was introduced into the BamHI site of Bluescript to generate pZSK-R. Dsrc41^{1/2} was inserted into the KpnI–PstI site of pZSK-R. The resultant Dsrc41^{1/2}–lacZ gene was introduced into pVH9, generating se^{Δ}.

Isolation of genomic DNA and cDNA clones

Genomic DNA and pupal cDNA clones of Dsrc41 were isolated from libraries described previously [Kojima et al. 1991]. For isolation of the first genomic DNA clone (ckg/k2), dtk5 [Shishido et al. 1991] was used as a probe. Hybridization was carried out at 55°C for 12 hr in H buffer (0.1 M Tris-HCl [pH 7.5], containing 1 M NaCl, 10 mM EDTA, 10 mM Denhardt’s solution, 0.2% SDS, and 200 μg/ml of heat-denatured salmon sperm DNA], which includes 32P-labeled probe DNA. Filters were washed at 55°C for 1–2 hr in 2× SSC (15 mM sodium citrate with 150 mM NaCl) containing 0.1% SDS. The second genomic DNA clone (kg/k2) was isolated using the cDNA insert of a ctk (see below) as a probe. cDNA clones were isolated using a 0.9-kb HindIII–Kpnl fragment of kg/k2 [see line A in Fig. 1A] as a probe. Hybridization was performed at 65°C for 12 hr in H buffer, including 32P-labeled, heat-denatured probe DNA. Filters were washed at 65°C for 1–2 hr in 0.1× SSC with 0.1% SDS.

Southern and Northern blotting

Canton-S DNA digested with suitable restriction enzymes was subjected to Southern analysis under less stringent conditions using a 0.9-kb HindIII–Kpnl fragment of kg/k2 as a probe. Filters were hybridized at 60°C in H buffer for 24 hr and then washed at 60°C in 2× SSC with 0.1% SDS for 1–2 hr. Northern hybridization was carried out as described previously [Kojima et al. 1991].

In vitro mutagenesis

Tyrosine 511 of Dsrc41 was replaced by a phenylalanine residue using PCR, in which one of the primers contained a mutated
sequence. With the introduction of the YF mutant, the following primers were used: 5'-GGGCCTACGACGGCGCAGATT-TCCC and 5'-AGTA-GGCCCTCCCTTGTGAGTC (underline, mutated base). The KR mutant was generated by the Kunkel method (Sambrook et al. 1989) using 5'-TTCAGGCT TCTAATTGCGC as the antisense primer. All other procedures for molecular cloning were as described by Sambrook et al. [1989].

Germ-line transformation

A mixture of 300 μg/ml of Dsrc41 plasmid DNA and 150 μg/ml of a helper plasmid, pUC282Δ-3, was injected into vกระడ, ryกระดย embryos (Spradling and Rubin 1982). Three WT-A (first chromosome), B (third), and C (first) and two YF-A (third) and B (first) viable transformant lines having wild type and activated Dsrc41, respectively, were obtained. The following were used as crosses to examine suppressor/mutant, respectively, were from Umea and Tubingen stock centers. Three [WT-A (first chromosome), B (third), and C (first)] and two [YF-A (third) and B (first)] viable transformant lines [KR-A (second), B (second), C (third), and D (third); DL-A (second) and B (second)] were obtained. In addition, two Dsrc41+/krff lines (KRYF-A and KRYF-B) were recovered.

Fly stocks

Canton-S [wild type] and vกระดย, ryกระดย were our laboratory stocks, and svpกระดย, an enhancer trap line of svp was obtained from G. Rubin [University of California, Berkeley]. A fly strain having a Dsrc41 deficiency [int2;R10voudec;Cy5;Gla] and fihกระดย, a DER mutant, respectively, were from Umea and Tubingen stock centers. The following were used as crosses to examine suppressor/enhancer activity: y; D-vel/Binsc.occ ptg (Tsuda et al. 1993); y; w; sp/Spx Dsor1+/Binsc.occ ptg (Tsuda et al. 1993); Sox152CyO [Rogge et al. 1991], y; E[sev]/1αneo/Binsc.occ ptg [Simon et al. 1991], E[sev]2B6α (dsrk)/CyO [Simon et al. 1991]; E[sev]3B7/TM6B, Tb [Simon et al. 1991]; Ras127/TM6B, Tb [Simon et al. 1991]; E[sev]3D09c/TM6B, Tb [Simon et al. 1991]; svp10; and boss’ h; ryกระดย [Kramer et al. 1991]. Enhancer trap lines H214 and AE127 (svp) were obtained from Y. Hiromi [Princeton University, NJ].

Immunohistochemistry and in situ hybridization

Immunostaining and in situ hybridization were performed essentially according to Higashijima et al. [1992] and Shishido et al. [1993]. Origins of anti-HRP, anti-lacZ (E. coli β-galactosidase) antibodies, and mAb23C10 were described in Higashijima et al. [1992]. Anti-Boss and anti-DE-cadherin antibody (DCAD2) were gifts of L. Zipursky [University of California, Los Angeles] and T. Uemura [Kyoto University, Japan]. A 1.9-kb EcoRI-Stu fragment of Dsrc41 cDNA was used as a template for making RNA probe.

Electron microscopy

Samples for electron microscopy were prepared and observed as described previously [Kojima et al. 1991].

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