A Drosophila SH2-SH3 Adaptor Protein Implicated in Coupling the Sevenless Tyrosine Kinase to an Activator of Ras Guanine Nucleotide Exchange, Sos

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Summary

A *Drosophila* gene (*drk*) encodes a widely expressed protein with a single SH2 domain and two flanking SH3 domains, which is homologous to the Sem-5 protein of *C. elegans* and mammalian GRB2. Genetic analysis suggests that *drk* function is essential for signaling by the sevenless receptor tyrosine kinase. *Drk* biological activity correlates with binding of its SH2 domain to activated receptor tyrosine kinases and concomitant localization of *drk* to the plasma membrane. In vitro, *drk* also binds directly to the C-terminal tail of Sos, a Ras guanine nucleotide-releasing protein (GNRP), which, like Ras1 and *drk*, is required for sevenless signaling. These results suggest that *drk* binds autophosphorylated receptor tyrosine kinases with its SH2 domain and the Sos GNRP through its Ski3 domains, thereby coupling receptor tyrosine kinases to Ras activation. The conservation of these signaling proteins during evolution indicates that this is a general mechanism for linking tyrosine kinases to Ras.

Introduction

Growth factor receptors with protein-tyrosine kinase activity mediate the biological effects of many of the polypeptide hormones that control cell proliferation, differentiation, and metabolism (Ullrich and Schlessinger, 1990; Pawson and Bernstein, 1990; Cantley et al., 1991). Cytoplasmic Src-like tyrosine kinases are also implicated in signal transduction processes, for example during T cell activation (Stein et al., 1992; Appleby et al., 1992; Strauss and Weiss, 1992; Abraham et al., 1992). Furthermore, a number of cellular and viral oncogenes encode constitutively active variants of

tyrosine kinases, which contribute to the development of specific cancers (Konopka et al., 1984; Hunter and Cooper, 1985). A principal intracellular signaling pathway by which tyrosine kinases stimulate cell growth and differentiation involves the activation of Ras guanine nucleotide-binding proteins, by conversion of Ras from a GDP- to a GTP-bound state. The notion that Ras might be important in propagating signals downstream of tyrosine kinases was originally suggested by the effects of microinjecting anti-Ras antibody into mammalian fibroblasts. Neutralizing antibody to Ras blocks the ability of both normal growth factor receptors and oncogenic tyrosine kinases to induce DNA synthesis (Mulcahy et al., 1985; Smith et al., 1986). Similarly, anti-Ras antibody and the S17N dominant inhibitory Ras protein inhibit the capacity of nerve growth factor, acting through the Trk tyrosine kinase, to induce neurite extension in PC12 neuronal cells (Szeberenyi et al., 1990; Hagag et al., 1986). Expression of S17N inhibitory Ras protein in PC12 cells or in fibroblasts also blocks the activation of serine/threonine kinases, such as Raf and MAP kinase, in response to nerve growth factor, platelet-derived growth factor, or insulin (Thomas et al., 1985; Wood et al., 1992; de Vries-Smits et al., 1992). These results indicate that Ras functions downstream of tyrosine kinases in mammalian cells to regulate Raf and the MAP kinase pathway.

The genetic dissection of signal transduction pathways in *Drosophila melanogaster* and *Caenorhabditis elegans* has indicated that receptor tyrosine kinases, as well as Ras and Raf proteins, also play an essential role in cell-cell communication during invertebrate development (Greenwald and Rubin, 1992; Horvitz and Sternberg, 1991; Dickson and Hafen, 1992; Dickson et al., 1992b). In *Drosophila*, the specification of R7 photoreceptor cells in the developing ommatidial units of the compound eye is dependent on the local activation of the sevenless (*sev*) receptor tyrosine kinase in the R7 precursor cell (Hafen et al., 1987). In the absence of functional *sev* protein, the R7 precursor does not develop into a photoreceptor cell, but rather into a nonneuronal cone cell (Tomlinson and Ready, 1986). Conversely, the constitutive activation of *sev* kinase activity in the R7 and other ommatidial precursor cells results in the formation of multiple R7 cells in each ommatidium (Basler et al., 1991). The response of ommatidial precursor cells to either a partially functional, or a constitutively active *sev* kinase is dosage sensitive. This observation has been exploited to identify genetically downstream components of the *sev* signaling pathway, establishing that *Gap1*, *Ras1*, and *Son of sevenless* (*Sos*), a putative guanine nucleotide-releasing protein (GNRP), play essential roles in signaling from the *sev* receptor (Rogge et al., 1992; Gaul et al., 1992; Sinion et al., 1991; Rogge et al., 1991). Similarly, in *C. elegans* it has been shown that a receptor tyrosine kinase encoded by the *let-23* gene and a Ras protein encoded by *let-60* are required for induction of the vulval cell fate (Aroian et al., 1990; Beitel et al., 1990; Han and Sternberg, 1990).

Ras proteins are biologically inactive in the GDP-bound state and are activated by the exchange of GDP for GTP (Bourne et al., 1991). In principle, Ras GTP binding could be induced either by stimulation of guanine nucleotide

exchange by a GNRP or by inhibition of a Ras GTPase-activating protein (GAP). In the *sev* pathway, it has been proposed that Ras1 activation is achieved both by stimulation of the Sos GNRP and by inhibition of Gap1 (Rogge et al., 1992; Gaul et al., 1992). Biochemical data from mammalian cells have shown that growth factors increase the fraction of Ras in the GTP-bound state (Gibbs et al., 1990; Satoh et al., 1990a, 1990b), and have suggested that regulation of GNRP activity may be the predominant mechanism by which growth factor receptors control Ras activation (Zhang et al., 1992; Medema et al., 1993). However, nothing is known about the mechanisms by which tyrosine kinases might communicate with a Ras GMRP.

Targets of receptor tyrosine kinases frequently possess Src homology 2 (SH2) domains that bind to specific phosphotyrosine-containing sites on activated receptors (Koch et al., 1991; Pawson and Gish, 1992). Such high affinity interactions appear important for the subsequent stimulation of SH2-containing cytoplasmic enzymes by tyrosine kinases, leading to the modulation of intracellular signaling pathways (Vega et al., 1992; Peters et al., 1992; Mohammadi et al., 1992). A majority of proteins with SH2 domains also contain one or more SH3 domains (Mayer et al., 1988; Musacchio et al., 1992a). While the biological functions of SH3 domains are uncertain, the Abl SH3 domain was recently shown to bind in vitro to a proline-rich sequence within a protein (3BP-1) that also contains a presumptive Rho/Rac GAP domain (Cicchetti et al., 1992). Although polypeptides with Ski2 and SH3 domains frequently also possess enzymatic domains, the products of the *sem-5/GRB2*, *crk*, and *nck* genes are composed almost exclusively of SH2 and SH3 domains (Clark et al., 1992; Lowenstein et al., 1992; Mayer et al., 1988; Lehmann et al., 1990). These proteins may serve as adaptors, by coupling tyrosine kinases to downstream targets that themselves do not contain SH2 or SH3 domains.

Genetic evidence from *C. elegans* indicates that the *sem-5* gene product, which contains a single SH2 domain flanked by two SH3 domains, functions on the same pathway as the Let-23 receptor tyrosine kinase and Let-601 Ras during induction of vulva1 differentiation (Clark et al., 1992). A mammalian homolog of *sem-5* has been identified, *GRB2*, suggesting that this pathway has been highly conserved in evolution (Lowenstein et al., 1992). If proteins with SH2 and SH3 domains also provide a link between the *sev* receptor tyrosine kinase and Ras in the R7 pathway, mutations in the corresponding genes might be expected to impair signaling from the receptor, as observed for mutations in *Sos* and *Ras1*. Here we describe the identification of a Drosophila homolog of *sem-5/GRB2*: *drk* (downstream of receptor kinases). Mutations at the *drk* locus have been identified in screens for second site modifiers of both loss- and gain-of-function *sev* alleles, implicating *drk* as an essential component of the *sev* signal transduction pathway. We present both genetic and biochemical evidence suggesting that this protein couples receptor tyrosine kinases through its SH2 and SH3 domains to Sos, a Ras GMRP.

Results

Isolation of a cDNA Encoding a Drosophila Homolog of *sem-5/GRB2*

To identify Drosophila cDNAs that might encode Ski2 domains, a cDNA expression library made from Drosophila embryos at 10-12 hr of development was screened using the CORT procedure (Skolnik et al., 1991; Margolis et al., 1992). The cDNA expression library was screened with a phosphotyrosine-containing C-terminal fragment of the activated human epidermal growth factor (EGF) receptor, which contains high affinity binding sites for the SH2 domains

of several mammalian signaling proteins (Margolis et al., 1990; Skolnik et al., 1991), including GRB2 (Lowenstein et al., 1992). We considered it possible that this phosphotyrosine-containing EGF receptor fragment would also bind the SH2 domains of Drosophila signaling proteins.

The cDNA insert of a single positive bacteriophage was sequenced and proved to encode a polypeptide sequence containing an SH2 domain. To obtain the complete coding sequence, the library was rescreened using this cDNA as a probe, leading to the isolation of an 1156 bp cDNA containing a single open reading frame. This latter cDNA is predicted to encode a protein of 211 amino acids, with a molecular mass of 24 kd (Figure 1A). Analysis of the *drk* protein sequence showed that it contains a centrally located SH2 domain, flanked by two SH3 domains (Figure 1B). The SH2 sequence possesses residues that are highly conserved among other SH2 domains (Koch et al., 1991), apparently because they are important for SH2 structure and function (Waksman et al., 1992; Overduin et al., 1992; Booker et al., 1992). The *drk* SH3 domains contain characteristic residues (Musacchio et al., 1992a), which from structural analysis of the spectrin and Src SH3 domains are involved in formation of the SH3 fold or in ligand binding (Yu et al., 1992; Musacchio et al., 1992b). The Drosophila *drk* polypeptide is most similar in both overall structure and amino acid sequence to the Sem-5 protein of *C. elegans* (61% identity) and human GRB2 (63% identity) (Figure 1B). These results suggest that *drk* is a Drosophila homolog of *sem-5/GRB2*.

Expression of *drk* RNA and Protein

To determine the cells in which *drk* is expressed during development, we performed in situ hybridization with digoxigenin-labeled probes to whole-mount imaginal discs of third instar larvae. As a control we employed a probe to the *sev* transcript, which is specifically expressed in a subpopulation of developing ommatidial cells behind the morphogenetic furrow (Figure 2A). In contrast with *sev*, we detected a strong, homogeneous labeling of all cells in the developing eye and antenna1 disc with the *drk* probe (Figure 2B). Widespread expression of *drk* was also observed in the other imaginal discs tested, as well as in the embryo (data not shown). It therefore appears that *drk* is broadly expressed in many different cell types.

To identify the *drk* protein, polyclonal antibodies were raised to the full-length *drk* polypeptide, synthesized in bacteria using the pET expression system. Affinity-purified anti-*drk* antibodies specifically recognized a 24 kd protein in Western blots of Drosophila extracts. The 24 kd *drk* protein was present in similar amounts in lysates from pupae, larvae, and adults (Figure 3). These results provide direct evidence that *drk* encodes a 24 kd polypeptide that is widely expressed throughout the Drosophila life cycle.

Figure 2. Comparison of the Distribution of *sev* and *drk* Transcripts in Eye imaginal Discs

(A) *sev* transcripts are only detected in a subpopulation of ornatidial precursor cells behind the morphogenetic furrow.

(B) High levels of *drk* transcripts are detected in all cells of the eye antennal disc. Scale bar, 40 μ m.

the *drk* gene. This suggested that the *Su(Sev^{S11})R1* and *E(sev)2B* mutations might lie within the *drk* gene.

The *Sev^{S11}* phenotype is caused by the overexpression of a truncated, activated *sev* kinase under the control of a duplicated *sev* enhancer (sE). We assume that the suppression of the *Sev^{S11}* phenotype in flies heterozygous for *Su(Sev^{S11})R1* is caused by a reduction in the amount of functional *Su(Sev^{S11})R1* gene product. If *drk* is indeed encoded by the *Su(Sev^{S11})R1* locus, it should be possible to revert the dominant suppression induced by this mutation by expressing the wild-type *drk* cDNA under control of the *sev* enhancer. For this purpose, we assembled a P element transformation construct, containing the *drk* cDNA joined to both the *hsp70* promoter and the *sev* enhancer sequences (*sE-hsp-drk*), linked to the white⁺ (*w*⁺) marker. Several independent transgenic lines were established with this construct. Since this transgene is expressed, in the absence of heat shock, in precisely the same cells as the constitutively active *Sev^{S11}* kinase, it should rescue the suppression of *Su(Sev^{S11})R1*. Indeed, one copy of the *sE-hsp-drk* construct was sufficient to revert completely the suppression of *Sev^{S11}* caused by the *Su(Sev^{S11})R1* or *E(sev)2B* allele (Figure 4C). These results indicate that the locus identified by the *Su(Sev^{S11})R1/E(sev)2B* mutations encodes *drk*.

Transheterozygous combinations of *E(sev)2B* and *Su(Sev^{S11})R1* cause pupal lethality, suggesting that the

drk gene plays an essential role in developmental processes other than R7 formation. To test whether the excessive lethality of *drk* mutations could be rescued by the ubiquitous expression of the *drk* cDNA, we crossed *E(sev)2B* CyO females with *Su(Sev^{S11})R1/Sco* males that contained the *sE-hsp-drk* construct. The progeny of these flies were subjected to repeated heat shock during development to induce general expression of the *sE-hsp-drk* transgene. In the absence of heat shock, *E(sev)2B/Su(Sev^{S11})R1* mutant animals containing the *sE-hsp-drk* transgene did not survive. However, if the cultures were heat shocked every 8 hr, the *E(sev)2B/Su(Sev^{S11})R1* mutant animals survived to adults. These animals constituted 18% (150 of 821) of the total progeny class, in good agreement with the value of 12.5% that might be expected from this cross. Surviving flies were fully viable and fertile, demonstrating that ubiquitous expression of *drk* can rescue the recessive lethality of *E(sev)2B/Su(Sev^{S11})R1* mutations

The *Su(Sev^{S11})R1* and *E(sev)2B* Mutations Affect Highly Conserved Drk SH2 Residues

Figure 3. Expression of the Drk Protein in Drosophila Tissues

Extracts of Drosophila larvae, pupae, and adult flies were separated by SDS-PAGE and subjected to Western blotting with preimmune serum or affinity-purified anti-drk antibodies, as indicated. The position of the 24 kd *drk* protein is indicated.

Figure 4. Expression of Drk under Control of the *sev* Enhancer Rescues the Dominant Suppression of the *Sev^{S11}*: Rough Eye Phenotype by *Su(Sev^{S11})R1*

Scanning electron micrographs and histological sections of the eyes of *Sev^{S11}* (P. and C), *Sev^{S11}: Su(Sev^{S11})R1/+* (B and E), and *Sev^{S11}/P(w⁺ sE-hsp-drk); Su(Sev^{S11})R1/+* (C and F) are shown. The overexpression of the N-terminally truncated *Sev^{S11}* protein under the control of the *sev* enhancer results in the constitutive activation of the *sev* kinase. This causes the recruitment of multiple R7 cells per ommatidium and concomitant roughening of the eye surface (A and D). Reducing the number of functional gene copies of *Su(Sev^{S11})R1* suppresses the formation of multiple R7 cells and the rough eye phenotype (B and E). This suppression is rescued by expression of the *drk* cDNA under the control of the *sev* enhancer (C and F), indicating that the *Su(Sev^{S11})R1* locus encodes the *drk* protein. Scale bar, 100 μ m in (C) and 10 μ m in (F).

The ability of the *drk* cDNA to suppress the phenotypic effects of the *Su(Sev^{S11})R1* and *E(sev)2B* mutations strongly suggested that these mutations might reside within the *drk* gene. We therefore analyzed the genomic sequence of *drk* from flies heterozygous for the *Su(Sev^{S11})R1* or *E(sev)2B* mutations (see Experimental Procedures). Figure 1C illustrates the genomic organization of *drk*. Sequence analysis revealed a guanine to adenine change in *Su(Sev^{S11})R1* (corresponding to nucleotide 500 in the cDNA sequence in Figure 1A), and acytosine to thymidine change in *E(sev)2B* (corresponding to nucleotide 616 in the cDNA sequence). Both these mutations are transitions, as expected from the mutagenic action of ethyl methanesulfonate. In each case the mutation resides within the sequence coding for the SH2 domain. Specifically, in the product of the *Su(Sev^{S11})R1* allele an arginine at position 67 is changed to a histidine, and in the *E(sev)2B* product a histidine at position 106 is changed to a tyrosine. These two residues are highly conserved among SH2 domains, and from X-ray crystallographic

analysis of the Src SH2 domain seem to play a critical role in both phosphotyrosine binding and also, in the case of the conserved histidine, in maintaining the overall structure of the SH2 domain (Waksman et al., 1992). These results confirm that the phenotypic effects caused by the *E(sev)2B* and *Su(Sev^{S11})R1* alleles are due to mutations in the *drk* gene.

The Drk Protein Binds to Receptor Tyrosine Kinases

The initial *drk* cDNA was isolated by binding of its encoded SH2 domain to the human EGF receptor. To confirm the ability of the *drk* protein to associate with activated receptor tyrosine kinases, *drk* was expressed in bacteria as a glutathione S-transferase (GST) fusion protein and bound to glutathione-agarose beads. The immobilized GST-*drk* fusion protein was then incubated with lysates of rat-1 fibroblasts engineered to overexpress the human EGF receptor. The GST-*drk* polypeptide formed a physical complex with the autophosphorylated EGF receptor from EGF-stimulated cells, but not from unstimulated cells (Figure 5A).

Figure 5. Binding of Wild-Type and Mutant Drk to Receptor Tyrosine Kinases

(A) Drk binding to human EGF receptor in vitro. R1hER cells were serum Starved: unstimulated cells (-) or cells stimulated with EGF (+) were lysed and incubated with GST, GST-drk (drk), or GST-drk^{E(sev)2B} (Drk 2B) fusion proteins immobilized on glutathione-agarose beads. EGF receptor association was detected by immunoblotting with anti-EGF receptor antibodies. The arrowhead indicates the position of the EGF receptor.

(B and C) Drk binding to Drosophila torso-sev chimeric receptor in vitro. Transgenic flies expressing the torso-sev chimeric protein under control of the *hsp70* promoter were uninduced (-) or heat shocked (+) and homogenized. Clarified lysates were incubated with immobilized GST, GST-drk (drk), or GST-drk^{E(sev)2B} fusion proteins, and associated torso-sev was identified by immunoblotting with anti-phosphotyrosine antibodies (B) or by subjecting the complexes to in vitro kinase reactions (C).

(D) Drk binding to torso-sev in vivo. Lysates of uninduced (-) or heat-shocked (+) flies were immunoprecipitated with anti-drk or anti-sev antibodies, and the immune complexes were subjected to in vitro kinase reactions. The mobility of torso-sev tyrosine kinase, detected by autophosphorylation, is indicated.

To examine the ability of drk to bind activated sev tyrosine kinase, a chimeric torso-sev polypeptide was inducibly expressed in transgenic *Drosophila* using the heat shock promoter. Torso-sev possesses a cytoplasmic sev tyrosine kinase domain, which is constitutively activated by its fusion to the extracellular domain of a gain-of-function mutant of the torso receptor tyrosine kinase (Sprenger and Nijsslein-Volhard, 1992; Dickson et al., 1992a). Immobilized GST-drk fusion protein was incubated with uninduced or heat-shocked *Drosophila* lysates, and the resulting complexes were immunoblotted with anti-phosphotyrosine antibodies to identify autophosphorylated torso-sev. The GST-drk fusion protein specifically bound tyrosine-phosphorylated torso-sev in lysates of heat-shocked flies (Figure 5B). Association of GST-drk with torso-sev from heat-shocked flies was also detected by incubating the complexes in kinase reactions to allow in vitro autophosphorylation of torso-sev (Figure 5C).

The association of drk with torso-sev in vivo was investigated by immunoprecipitating lysates of uninduced or heat-shocked flies with anti-drk or anti-sev antibodies. Torso-sev was identified in these immunoprecipitates by its ability to autophosphorylate in vitro. The torso-sev tyrosine kinase was present in both anti-sev and anti-drk immunoprecipitates from heat-shocked flies (Figure 5D). Similar results were obtained by immunoblotting these immunoprecipitates with anti-phosphotyrosine or anti-sev antibodies (data not shown). These results indicate that wild-type drk associates with the activated torso-sev tyrosine kinase in flies.

The effect of the amino acid substitution (His-106→Tyr) introduced into the drk SH2 domain by the *E(sev)2B* mutation was investigated by incorporating this change into the GST-drk fusion protein, and examining its effect

on receptor binding. Unlike the wild-type drk polypeptide, the mutant protein failed to bind in vitro to either the autophosphorylated EGF receptor (Figure 5A) or the activated torso-sev chimeric receptor (Figures 5B and 5C). The ability of drk to bind autophosphorylated receptors is therefore compromised by the substitution in the SH2 domain induced by the *E(sev)2B* mutation.

An Amino Acid Substitution in the SH2 Domain Alters the Subcellular Localization of the Drk Protein

The results presented above suggest that drk functions in the development of photoreceptors, and at other stages of *Drosophila* development, to couple receptor tyrosine kinases to one or more downstream signaling pathways. To investigate the expression of drk protein in ommatidial precursor cells, whose development is obstructed by the *Su(Sev)^{S11}R1* and *E(sev)2B* mutations, wild-type *Drosophila* eye imaginal discs were immunostained with affinity purified anti-drk antibodies. As was expected from the in situ localization of RNA transcripts, the drk protein was expressed at high levels in all cells of the eye antennal disc. drk was localized at the plasma membrane in the apical portions of the epithelial imaginal disc cells. Figure 6A shows a projection of a series of confocal sections through an eye imaginal disc, probed with anti-drk antibodies. Immunofluorescent staining was primarily observed at the outer membrane of all cells in the imaginal disc. In the cells of the epithelium proper, the most intense staining was observed in the most apical 5 μm of each cell. In the morphogenetic furrow (Figure 6A, black arrow) the diameters of the cells are reduced, owing to the apical constriction associated with the moving furrow. Behind the morphogenetic furrow, a consistently higher level of staining was observed in a subpopulation of developing

Figure 6. Localization of Wild-Type and Mutant Drk Protein in Eye Imaginal Discs

Projections of optical sections of eye imaginal discs of wild-type (A and C) and *E(sev)2B* mutant larvae (B and D) stained with affinity-purified anti-drk antibodies are shown. Wild-type drk protein is highly concentrated at the apical membrane of all imaginal disc cells. In the morphogenetic furrow (black arrow in [A]) the density of ommatidial cells is higher, owing to their apical constriction in the furrow. Behind the morphogenetic furrow drk marks the assembly of the ommatidial clusters and is expressed at higher levels in a subpopulation of developing photoreceptor cells (C). The *drk^{E(sev)2B}* protein, that carries a histidine to tyrosine amino acid substitution in the SH2 domain, is expressed at similar levels as the wild-type protein but is not associated with the cell membrane. Rather, it is localized in the cytoplasm. The black arrow head in (B) indicates the cytoplasmic drk staining in the flat cells of the peripodial membrane. Since drk is required for imaginal disc development, ommatidial assembly in *E(sev)2B* discs is abnormal. However, higher levels of mutant protein are still observed in cell clusters behind the morphogenetic furrow (white arrow in [B]). Scale bar, 10 μ m.

photoreceptor cells. A higher magnification of anti-drk labeling is shown in Figure 6C.

Since the *sev* receptor tyrosine kinase is also restricted to the cell membrane in the apical region of a subpopulation of developing ommatidial cells, the physical association of drk and *sev*, demonstrated above, might contribute to drk localization in these cells. Clearly, however, the membrane localization of drk is not exclusively due to *sev* binding since *sev* is only expressed transiently in a small subpopulation of imaginal disc cells and is only activated in a single cell, the R7 precursor. Furthermore, no alteration in the membrane localization of the drk protein was observed in eye discs derived from *sev* mutant larvae (data not shown).

The involvement of the drk Ski2 domain in localization to the membrane could be directly tested, since drk encoded by the mutant *E(sev)2B* allele contains an amino acid substitution of a highly conserved residue required for phosphotyrosine binding. Animals homozygous for *drk^{E(sev)2B}* die at the pupal stage: imaginal discs of homozygous *drk^{E(sev)2B}* larvae reduced in size, and the

assembly of the ommatidial clusters is abnormal. Compared with anti-drk staining of the wild-type disc, no appreciable loss in the overall intensity of immunofluorescence was observed in the *drk^{E(sev)2B}* homozygous mutant discs. However, the pronounced straining of the apical membrane portions of each cell, observed in wild-type eye discs, was not detected in mutant discs. In contrast, the immunofluorescence was more cytoplasmic (Figure 6B), as evident in the flat cells of the peripodial membrane (black arrowhead in Figure 6B). Although ommatidial assembly in *E(sev)2B* flies is abnormal, higher levels of mutant drk protein are still observed behind the morphogenetic furrow (Figure 6D). These results suggest that localization of the drk protein to the apical portion of the cell membrane requires a functional SH2 domain. These observations are consistent with the view that drk is recruited to the plasma membrane by binding of its SH2 domain to membrane associated phosphotyrosine-containing proteins, such as activated receptor tyrosine kinases.

Drk Binds to the C-Terminal Tail of Sos

The identification of drk as a gene required for sev signaling, and the ability of the drk SH2 domain to bind activated receptor tyrosine kinases, suggests that the drk protein is a target of sev. Other loci, including *Sos* and *Ras1*, have been identified in genetic screens as being required for signaling downstream of sev (Simon et al., 1991; Rogge et al., 1991). *Sos* encodes a polypeptide with a centrally located domain related in sequence to the CDC25 gene product of *Saccharomyces cerevisiae*, which catalyzes the exchange of GDP for GTP on Ras, thereby leading to Ras activation. Genetic analysis suggests that the *Sos* protein may act downstream of sev as a GNRP for Ras1 during *Drosophila* development. Given that drk also lies in this pathway and can bind directly to activated receptors, it is possible that drk links receptor tyrosine kinases to *Sos*, which in turn controls the activation of Ras1. If the drk SH2 domain mediates its interactions with activated receptors, then the SH3 domains, which make up the remainder of the protein, are likely to provide binding sites for targets immediately downstream of drk. Recent evidence has suggested that the SH3 domains of Abl and Src can bind to a proline-rich motif in the protein 3BP-1 (APTMPPLPP), containing the sequence PPPLPP (Cicchetti et al., 1992; Ren et al., 1993). nuclear magnetic resonance analysis of the Src SH3 domain complexed with a peptide corresponding to the 3BP-1 SH3-binding site suggests that these prolines may directly contact the SH3 domain (Yu et al., 1992). In *Sos*, the 505 amino acid sequence C-terminal to the CDC25-like domain is relatively rich in proline residues (15%) (Simon et al., 1991), and contains several sequences related to the 3BP-1 SH3-binding site (Figure 7A).

We therefore tested the ability of drk to associate physically with *Sos*. Bacterial GST-*Sos* fusion proteins were expressed that contained either residues 1225-1405 within the C-terminal tail of *Sos* (*Sos* tail), including three of the proline-rich sequences noted in Figure 7A, or residues 505-692 from the amino-terminal region (*Sos* N). Lysates of bacteria expressing the parental GST protein, or the GST-*Sos* fusion proteins, were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter. The filter was then incubated with drk protein purified from bacteria. Drk bound specifically to the immobilized GST-*Sos* tail protein, but did not recognize GST or the GST-*Sos* N polypeptides, nor any other bacterial proteins (Figure 7B). In a complementary experiment, the GST-*Sos* tail protein, immobilized on beads, bound specifically to drk in *Drosophila* lysates (Figure 7C). These results demonstrate that drk has an intrinsic affinity for the C-terminal tail of *Sos*.

Discussion

A Protein with St12 and SH3 Domains Functions in Signal Transduction during *Drosophila* Development

We have isolated a *Drosophila* cDNA encoding a protein with the structure SH3-SH2-SH3, and have identified this gene product as the *Drosophila* homolog of Sem-5 and GRB2. We have used both genetic and biochemical techniques to investigate the role of drk in the sev receptor tyrosine kinase signal transduction pathway, which controls the specification of R7 photoreceptor cell fate.

Figure 7. Drk Binds to the Tail of *Sos*

(A) The C-terminal tail of *Sos* contains potential SH3-binding sites. A sequence encompassing the Abl SH3-binding site in the protein 3BP-1 is compared with motifs in the C-terminal region of *Sos*. A proline-rich element, postulated to occupy the SH3 ligand-binding site, is boxed. Basic residues following the proline-rich elements of *Sos* are also outlined.

(B) Bacterial lysates expressing GST, GST-*Sos* N, and GST-*Sos* tail were fractionated by SDS-PAGE and transferred to nitrocellulose. This filter was then incubated with purified bacterial drk protein. Binding of drk was detected by immunoblotting with anti-drk antibodies. An arrow indicates the mobility of the GST-*Sos* tail.

(C) Protein lysates of torso-sev transgenic *Drosophila*, either uninduced (-) or heat shocked (+), were incubated with immobilized GST-*Sos* tail, and complexes were analyzed by immunoblotting with anti-drk antibodies. The position of drk is indicated.

Simon et al. (1993 [this issue of Cell]) have also identified the drk gene and have demonstrated its involvement in signaling between sev and Ras1.

The cytological map position of the gene encoding the drk protein suggested that it might correspond to the *E(sev)2B/Su(Sev^{S11})R1* locus, in which loss-of-function mutations have been identified either as dominant enhancers of a weak sev mutation or as suppressors of the gain-of-function mutation *Sev^{S11}*. Indeed, expression of the drk cDNA under the control of the sev enhancer and heat shock promoter rescued the dominant suppression of the *Sev^{S11}* phenotype by the *Su(Sev^{S11})R1* mutation. Repeated heat shock induction of drk rescued the lethality of combined *E(sev)2B/Su(Sev^{S11})R1* mutations. Previous genetic analysis has indicated that the product of this gene is required for signaling by both the sev and DER receptor tyrosine kinases (Simon et al., 1991). Although embryos lacking DER function do not develop to the larval stage (Schejter and Shilo, 1989; Price et al., 1989), loss of drk function results in pupal lethality. It is possible that drk mutant larvae can survive to the pupal stage in the absence of zygotically produced protein owing to maternal product that is deposited in the egg. Consistent with this hypothesis, a relatively high level of drk RNA can be detected by in situ hybridization in unfertilized eggs (data not shown). Furthermore, it has been shown that drk function is required maternally for signaling from the torso

receptor tyrosine kinase during early embryonic development (Doyle and Bishop, 1993). Therefore the drk protein appears to serve an essential function in the signal transduction pathways activated by different receptor tyrosine kinases.

The SH2 Domain Links Drk to Activated Receptor Tyrosine Kinases

Sequencing of the drk gene from *Drosophila* carrying the *E(sev)2B* or *Su(Sev)^{S11}R1* alleles identified mutations in each allele, both of which introduce amino acid substitutions into the drk SH2 domain. On purely theoretical grounds, both of these substitutions would be expected to impair the ability of the drk SH2 domain to bind to phosphotyrosine-containing polypeptides. In the *E(sev)2B* product His-106 is changed to tyrosine. This histidine (His β D4 by the nomenclature of Eck et al. [1993]), is conserved in all but one of the known SH2 domains (Koch et al., 1991), and the homologous residue in the Src SH2 domain (His-201 of the Src protein) plays a crucial role in forming the phosphotyrosine-binding site. The X-ray crystallographic structure of the Src SH2 domain bound to phosphotyrosine-containing peptides reveals that the His-201 side chain interacts with phosphotyrosine (J. Kuriyan, personal communication). His-201 serves as a platform to position Arg-155 (Arg α A2), which also makes direct contact with the phosphotyrosine (Waksman et al., 1992). In addition, His-201 forms hydrogen bonds with Glu-159 (Glu α A6), in the first α helix, and with Ser-187 (Ser pC5) in the third β strand. Hence, His-201 appears to be important in dictating the overall structure of the Src SH2 domain, as well as in forming the phosphotyrosine-binding site. Consistent with this view, incorporation of the *E(sev)2B* mutation into the GST-drk fusion protein abrogated its ability to bind the activated torso-sev tyrosine kinase and autophosphorylated EGF receptor in vitro. We have previously noted that substitution of the homologous histidine in the N-terminal GAP SH2 domain compromises its binding to growth factor receptors in vitro (Marengere and Pawson, 1992).

The *Su(Sev)^{S11}R1* mutation also affects a highly conserved residue in the drk SH2 domain (Arg-67), which corresponds to Arg-155 of Src (Arg α A2). In the Src SH2 domain, this residue forms an important element of the phosphotyrosine-binding site. A nitrogen of the terminal guanidinium group interacts with the electron density of the tyrosine ring, while the intrachain (c) nitrogen makes a hydrogen bond with a phosphate oxygen (Waksman et al., 1992).

These results indicate that the ability of the drk SH2 domain to recognize phosphotyrosine is essential for drk biological activity. It is of interest to note that the mammalian homolog of drk, GRB2, is not normally a substrate for tyrosine phosphorylation, even in cells stimulated with growth factors or transformed with oncogenic tyrosine kinases (Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). Similarly, drk immunoprecipitated from lysates of *Drosophila* expressing the torso-sev tyrosine kinase did not contain detectable phosphotyrosine (data not shown). It is therefore likely that the function of GRB2 and drk is regulated not by phosphorylation, but by direct binding of their SH2 domain to autophosphorylated receptors or other phosphotyrosine-containing proteins, such as the SHC gene products (Rozakis-Adcock et al., 1992).

Immunostaining of imaginal discs from wild-type and *E(sev)2B* mutant larvae shows that the wild-type drk protein is predominantly associated with the apical membrane of imaginal disc cells, whereas the mutant drk protein, lacking a functional SH2 domain, is localized in the cytoplasm. Hence, receptor autophosphorylation may serve as a means to recruit drk, and any drk-associated proteins, to their presumed site of action at the membrane.

Drk Is Implicated in Coupling Receptor Tyrosine Kinases to Sos

The identification of Sos and Ras1 as genes required for sev signaling suggested the possibility that drk might couple receptor tyrosine kinases to Sos, which has a central domain related to CDC25, and is therefore likely to act as a Ras GNRP. The tail of Sos, C-terminal to the CDC25 domain, has several proline-rich sequences related to the SH3-binding site identified in 3BP-1, which might serve as contact sites for the SH3 domains of drk. Indeed, we found that purified drk bound specifically to the C-terminal tail of Sos in vitro.

These results suggest that one function of drk is to couple activated receptor tyrosine kinases, which it binds through its SH2 domain, to Sos, which it complexes with its SH3 domains. Indeed, substitution of a conserved proline in the C-terminal drk SH3 domain reduced its ability to bind the Sos tail (J. P. O., unpublished data). The provision of drk with two SH3 domains may increase its affinity for Sos, if these domains bind cooperatively to sites within the Sos tail. The Sos GNRP, in turn, can potentially activate Ras1 by catalyzing the exchange of GDP for GTP (Figure 8). This scheme proposes that the binding of the drk SH2 domain to autophosphorylated receptors serves to activate Sos guanine nucleotide exchange activity in vivo. This might be achieved simply by relocalizing drk associated Sos to the membrane, where Ras proteins are located. It is also possible that Sos activity is stimulated following binding of drk to a receptor, either by a conformational change transmitted through the SH3 domains or by tyrosine phosphorylation of Sos.

Recently, two mouse cDNAs encoding proteins very closely related to *Drosophila* Sos have been isolated (Bowtell et al., 1992). The mouse Sos1 and Sos2 proteins both have proline-rich tails with multiple potential SH3-binding sites. It is of interest that in the Sos proteins from both *Drosophila* and mouse, the C-terminal proline-rich sequences are invariably followed by one or more basic residues (usually an arginine; see Figure 7A), which might provide an element of specificity for the GRB2/drk SH3 domains. Given the evolutionary conservation of tyrosine kinases, the drk/Sem-5/GRB2 family, Sos, and Ras, it seems probable that the model outlined in Figure 8 will prove to be a general mechanism by which receptor tyrosine kinases communicate with Ras proteins.

Experimental Procedures

Isolation and Analysis of cDNA Clones

The intracellular domain of the human EGF receptor produced in a recombinant baculovirus expression system was autophosphorylated using [γ -³²P]ATP, followed by cleavage with cyanogen bromide as previously described (Skolnik et al., 1991; Margolis et al., 1992). The ³²P-radiolabeled carboxy-terminal EGF receptor tail was then used to probe a *Drosophila* 10-12 hr embryonic cDNA expression library (Novagen), constructed in the Ex-Lox bacteriophage vector as previously described (Margolis et al., 1992). Out of approximately 1.4 x 10⁶ phage screened, one clone (dAA) was isolated through the ability

Figure 8. A Model for the Role of Drk in Coupling-Activated Receptor Tyrosine Kinases to Sos

Drk is illustrated as binding to a phosphotyrosine-containing site on an activated receptor through its SH2 domain and to proline-rich motifs (PRO) in the tail of Sos through its SH3 domains. This interaction may serve to localize Sos to the membrane, where it can gain access to GDP-bound Ras, and might also stimulate Sos activity in vivo.

of its product to bind to the EGF receptor tail. Additional overlapping drk cDNA clones were obtained by screening the same library with clone dAA as a probe using standard DNA hybridization protocols. DNA sequence analysis was performed on plasmid subclones using the Sequenase system (United States Biochemical Corporation).

Polymerase Chain Reaction Amplification and Sequence Analysis of *drk* Genomic DNA

Polymerase chain reaction (PCR) amplification was performed on genomic DNA extracted from *Drosophila* heterozygous for either the *Su(Sev)²¹¹R1* or the *E(sev)2B* mutations, as well as from the parental strain w1118, *CyO/Sco*. The *drk* genomic locus was too large to amplify in a single PCR. Oligonucleotide primers derived from the drk cDNA sequence and based on the genomic organization of sem-5 (Clark et al., 1992) were therefore synthesized. Three sets of primers, D1 (GCATCAAGGCTGATAG) and D2 (TTGCAGTGGCCCTTCAGTGTG), D3 (GCAGGGGTAGAAGATTTAG) and D4 (AATTACTAGGTATGAG), and D5 (TCAAAATTGGTATCGCGCGGAG) and D6 (TTATGATGATATGG), were used to amplify the *drk* locus (Figure 1C). Each reaction was done in duplicate to minimize PCR artifacts. All PCRs were carried out under the following conditions: 100 ng of genomic DNA and 500 ng of each primer were denatured at 94°C, annealed at 55°C, and polymerized with pfu (Stratagene) at 72°C for 30 cycles on a Perkin Elmer thermal cycler. The resulting PCR products were subcloned into the pCR11 vector (Invitrogen), and multiple clones were sequenced.

5' sequences of wild-type genomic drk were obtained from cloned DNA, isolated by screening a *Drosophila* genomic library constructed in λ DASHIII (Stratagene) (kindly provided by M. Noll) with a full-length drk cDNA. Plaque hybridization and analysis of the positive phage clones were done according to standard protocols.

In Situ Hybridization

Digoxigenin-labeled DNA probes were prepared by random priming according to the Boehringer Mannheim Application Manual, with the exception that a 20-fold higher hexanucleotide concentration was used. Eye imaginal discs were dissected in Ringer's and fixed for 15 min in 4% paraformaldehyde. 0.2% Tween-20, 0.2% sodium deoxycholate. Hybridization and detection were carried out as described (Tautz and Pfeifle, 1989). The discs were mounted in Moviol-2.5% DAPCO.

A similar probe was used for in situ hybridization to polytene chromosomes (Ashburner, 1989).

Generation of Antibodies

Full length drk cDNA was cloned into the pET11d expression vector (Novagen) and into the pGEX2T vector (Pharmacia) to make nonfusion and GST fusion proteins, respectively. PCR was used to create restriction endonuclease sites to facilitate cloning of drk coding regions into the expression vectors. Specifically, NcoI (5') and BamHI (3') sites were employed to clone drk into pET11d, whereas BamHI (5') and EcoRI (3') sites were added to clone drk into pGEX2T. The resulting PET-drk clone was transformed into BL21 DE3 *Escherichia coli* and pGEX-drk was transformed into DH5 α *E. coli*. Recombinant protein was induced for 4 hr with 1 mM isopropyl- β -D-thiogalactopyranoside,

starting when the bacterial culture reached an OD₆₀₀ of 0.5-0.8. Bacterial pellets were sonicated three times for 20 s each in phospholipase C lysis buffer (50 mM HEPES [pH 7.5], 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM magnesium chloride, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 10 μ g/ml aprotinin and leupeptin) and clarified by centrifugation. The clarified lysate of bacteria induced to express the PET-drk clone was boiled in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS [w/v], 10% glycerol, and 5% β -mercaptoethanol) and separated by electrophoresis on a preparative SDS-polyacrylamide (15%) gel. The resulting nonfusion drk protein was excised, electroeluted, and used to generate rabbit polyclonal antibodies (anti-drk). GST-drk fusion protein was purified using glutathione-agarose and eluted with 10 mM glutathione as described previously (Pellicci et al., 1992). The rabbit polyclonal antibody was affinity purified from crude sera using purified GST-drk protein. Anti-sev antiserum (G39-20) was directed against the C-terminal portion of sev. Polyclonal rabbit anti-EGF receptor antibody has been described (Honegger et al., 1989).

Western Blotting and Immunoprecipitation

For immunoblotting of total *Drosophila* protein lysates, adult flies. Pupae or larvae were homogenized in SDS sample buffer. Rat fibroblasts overexpressing human EGF receptor (R1hER) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For growth factor stimulation, cells were serum starved for 24 hr in DMEM + 0.5% fetal calf serum and then stimulated with 80 nM EGF for 5 min and harvested. For immunoprecipitations, adult flies were homogenized, or R1hER cells were lysed in phospholipase C-LB containing 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation and incubated with 2 μ g/ml affinity-purified anti-drk, or a 1:200 dilution of anti-sev, and protein A-Sepharose, for 90 min at 4°C. Immunoprecipitates were washed three times in 1 ml of HNTG (20 mM HEPES [pH 7.5], 150 mM sodium chloride, 10% glycerol, 0.1% Triton X-100, and 1 mM sodium orthovanadate), and then boiled for 5 min in SDS sample buffer. All samples were fractionated by electrophoresis on 7.5%, 10%, or 15% SDS-polyacrylamide gels and transferred to nitrocellulose in a semidry blotting apparatus at 0.8 mA/cm² for 1 hr. Filters were blocked for 1 hr at room temperature in 5% nonfat dry milk in TBS-T (20 mM Tris [pH 7.5], 150 mM sodium chloride, and 0.05% Triton X-100) and probed with 2 μ g/ml affinity-purified anti-drk or a 1:1000 dilution of anti-sev. For anti-phosphotyrosine immunoblots, filters were blocked in 5% bovine serum albumin, 1% ovalbumin in TBS-T, and probed with 1 μ g/ml affinity-purified anti-phosphotyrosine antibodies. Anti-drk and anti-sev immunoblots were developed using either the epichemiluminescence kit (Amersham), alkaline phosphatase (Bio-Rad), or ¹²⁵I-labeled protein A (Amersham). The anti-phosphotyrosine immunoblots were processed using ¹²⁵I-labeled protein A.

In Vitro Binding Studies

The *E(sev)2B* mutation was introduced into the drk cDNA by PCR mutagenesis as described (Marengere and Pawson, 1992) and subcloned into the pGEX2T vector as above. The GST, GST-drk, or GST-drk^{E(sev)2B} fusion proteins were induced and purified by binding to glutathione-agarose beads. R1hER cells or adult flies were lysed in phospholipase C-LB, clarified and incubated with immobilized GST or GST fusion proteins for 90 min at 4°C. The protein complexes were washed three times with HNTG and boiled for 5 min in SDS sample buffer. Complexes were then resolved by SDS-PAGE and transferred to nitrocellulose. Filters were blocked and probed with antibodies as described above.

Protein complexes were also subjected to in vitro kinase assays. Both immune complexes and GST fusion protein complexes were incubated with 0.5 μ Ci of [γ -³²P]ATP in KRB (20 mM-HEPES [pH 7.4], 25 mM magnesium chloride, .4 mM manganous chloride, and 0.1 mM sodium orthovanadate) at room temperature for 15 min. Reactions were stopped by adding an equal volume of 2 x SDS sample buffer. A fraction of the kinase reaction was separated by SDS-PAGE, and the gel was fixed, dried, and exposed to film.

Two regions of Sos were subcloned by PCR into the pGEX2T vector: pGEX Sos N encodes amino acids 505-692 and pGEX Sos tail encodes amino acids 1225-1405 of Sos. Crude bacterial extracts expressing GST, GST-Sos N, and GST-Sos tail bacterial fusion proteins were resolved by 10% SDS-PAGE and were transferred to nitrocellulose. Filters were blocked in TBS containing 5% bovine serum albumin, 10/0 ovalbumin, and 0.1 % SDS for 1 hr. These filters were probed using purified drk protein from bacteria expressing drk for 3 hr at room temperature and washed three times with TBS plus 0.1%

SDS. Complexes were detected by immunoblotting using affinity-purified anti-drk antibodies.

Immunohistochemistry and Confocal Microscopy

Eye imaginal discs from late third instar larvae were dissected in Ringer's and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), 0.07 M glucose, 0.07 M CaCl₂ for 30 min. The discs were washed in phosphate-buffered saline-0.2% saponin, blocked for 30 min in phosphate-buffered saline-0.2% saponin containing 3% fetal bovine serum, and incubated overnight with affinity-purified anti-drk antibodies at 4°C. The discs were then washed three times with phosphate-buffered saline-0.2% saponin and then incubated with a fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:80; Southern Biotech, Inc.). After washing in phosphate-buffered saline-0.2% saponin, the discs were mounted in 70% glycerol containing 2.5% DAPCO.

Optical sections of immunofluorescence were obtained using a confocal laser scanning microscope (Multiprobe 2001, Molecular Dynamics). Pictures were prepared by using the maximal intensity projection option (Imagingspace software, Molecular Dynamics); photographs were taken off the screen.

Drosophila Strains

The genetic screen to identify suppressors of the *Sev^{S11}* phenotype will be described in detail elsewhere (T. R. et al., unpublished data). In brief, males were mutagenized with ethyl methanesulfonate or by the mobilization of P elements and crossed with females that were homozygous for a *Sev^{S11}* construct on the third chromosome (Basler et al., 1991). The progeny were scored for individuals that exhibit a suppression of the rough eye phenotype. *Su(Sev^{S11})R1* was mapped meiotically using the multiply marked chromosome *b pr cn bw*. *Su(Sev^{S11})R1* was shown to be allelic to *E(sev)2B* by complementation analysis. The chromosomal sites of integration of the P elements were determined by in situ hybridization with a biotin-labeled P element-specific probe as described by Easler and Hafen (1988).

Flies carrying a transgene encoding an activated torso-sev fusion protein (a fusion of the extracellular domain of torso⁴⁰²¹ and the intracellular kinase domain of sev) under the control of the *hsp70* promoter in the pW8 transformation vector (Dickson et al., 1992a) were stimulated at 37°C for 30 min and then allowed to recover at room temperature for 4 hr.

Rescue Construct and Germline Transformation

The *sE-hsp-drk* construct was made by inserting the full-length *drk* cDNA into a modified pW8 transformation vector (Klemenz et al., 1987), containing the *hsp70* promoter and a single copy of the 1.2 kb *sE* fragment (Basler et al., 1991). Plasmid DNA was prepared for injection as described previously (Basler et al., 1991). The *sE-hsp-drk* construct was injected into W1118 host embryos. Nine independent transformed lines were established. To test whether ubiquitous expression of the *drk* cDNA could rescue the lethality of *drk* mutations, *Su(Sev^{S11})R1/E(sev)2B*; *sE-hsp-drk* transformant flies were shifted to 37°C for 40 min every 8 hr during development.

Scanning Electron Microscopy and Histology

Adult flies for scanning electron microscopy were stored in 70% acetone before they were critical-point dried and coated for examination with a Hitachi S-4000 scanning electron microscope. Fixation and preparation of histological sections for light microscopy were performed as described previously (Basler and Hafen, 1988).

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