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Biochemical and genetic analysis of the Drk SH2/SH3 adaptor protein of *Drosophila*

Thomas Raabe[1,2], Jean Paul Olivier[3], Barry Dickson[1,4], Xiangdong Liu[1], Gerald D.Gish[3], Tony Pawson[3] and Ernst Hafen[1]

[1]Zoologisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland and [3]Programme in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario M5G 1X5, Canada
[2]Present address: Theodor-Boveri-Institut, Universität Würzburg, Lehrstuhl für Genetik, Am Hubland, D-97074 Würzburg, Germany
[4]Present address: HHMI, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

T.Raabe and J.P.Olivier contributed equally to this work

The Drk SH3-SH2-SH3 adaptor protein has been genetically identified in a screen for rate-limiting components acting downstream of the Sevenless (Sev) receptor tyrosine kinase in the developing eye of *Drosophila*. It provides a link between the activated Sev receptor and Sos, a guanine nucleotide release factor that activates Ras1. We have used a combined biochemical and genetic approach to study the interactions between Sev, Drk and Sos. We show that Tyr2546 in the cytoplasmic tail of Sev is required for Drk binding, probably because it provides a recognition site for the Drk SH2 domain. Interestingly, a mutation at this site does not completely block Sev function *in vivo*. This may suggest that Sev can signal in a Drk-independent, parallel pathway or that Drk can also bind to an intermediate docking protein. Analysis of the Drk–Sos interaction has identified a high affinity binding site for Drk SH3 domains in the Sos tail. We show that the N-terminal Drk SH3 domain is primarily responsible for binding to the tail of Sos *in vitro*, and for signalling to Ras *in vivo*.

Key words: *Drosophila*/Sevenless/signal transduction/Sos/SH2 adaptor protein/SH3 domain

Introduction

Receptor tyrosine kinases (RTKs) play an important role in mediating the responses of cells to extracellular signals during growth and differentiation of multicellular organisms. Ligand-induced dimerization of receptors leads to the activation of their kinase domain, and subsequent autophosphorylation (Schlessinger and Ullrich, 1992). The autophosphorylated receptor recruits a number of cytoplasmic proteins which contain a common sequence motif, the SH2 (src-homology 2) domain (Koch *et al.*, 1991; Pawson and Gish, 1992). SH2 domains bind to specific phosphotyrosine-containing peptide sequences (Songyang *et al.*, 1993; Marengere *et al.*, 1994) and hence may couple the stimulation of RTKs by their ligands to the activation of different cytoplasmic signalling pathways. Biochemical studies in mammalian systems and genetic analysis in *Drosophila* and *Caenorhabditis elegans* have indicated that one of these SH2 domain-containing proteins, called Grb2 in mammals (Lowenstein *et al.*, 1992), Sem-5 in *C.elegans* (Clark *et al.*, 1992) and Drk in *Drosophila* (Olivier *et al.*, 1993; Simon *et al.*, 1993) provides a functional link between RTKs and activation of the Ras protein. The Sem-5/Grb2/Drk proteins contain a single SH2 domain flanked by two SH3 (src-homology 3) domains. Binding of these polypeptides to autophosphorylated RTKs is mediated by the SH2 domain (Lowenstein *et al.*, 1992; Olivier *et al.*, 1993). Grb2 and Drk complex with the Ras guanine nucleotide exchange factor Sos through binding of their SH3 domains to proline-rich motifs in Sos (Buday and Downward 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). Both genetic and biochemical studies have shown that activation of Ras by RTKs stimulates a conserved kinase cascade consisting of Raf1, MAP kinase kinase (MAPKK) and MAP kinase (MAPK) (for a recent review, see Marshall, 1994).

In *Drosophila*, mutations in *drk* impair signalling via the Sevenless (Sev) receptor tyrosine kinase (Simon *et al.*, 1991, 1993; Olivier *et al.*, 1993). The *sev* gene is expressed in a subset of the 20 cells that give rise to the ommatidial units of the compound eye (Tomlinson *et al.*, 1987). In the wild type, the initiation of photoreceptor cell development in the R7 precursor cell is dependent on the local activation of Sev by its ligand, the Boss protein, which is expressed on the neighbouring R8 cell (Krämer *et al.*, 1991). In the absence of a functional Sev or Boss protein, the R7 precursor cell differentiates as a non-neural cone cell (Tomlinson and Ready, 1986; Reinke and Zipursky, 1988). Conversely, if the Sev RTK is constitutively activated by a mutation and overexpressed in the same subset of cells as the wild-type protein, not only the R7 precursor but also the cone cells initiate neural development (Basler *et al.*, 1991; Dickson *et al.*, 1992). The precursors of the R7 and the cone cells are often referred to as the R7 equivalence group (Greenwald and Rubin, 1992). The excess recruitment of R7 cells in *sev* gain-of-function mutations, or the frequent loss of R7 cells in weak *sev* mutations, provide genetically sensitized systems that have been used to screen for second-site modifiers (Simon *et al.*, 1991; Olivier *et al.*, 1993; T.R. and E.H., unpublished data). Loss-of-function mutations of *drk* are homozygous lethal, but in the heterozygous condition suppress the recruitment of multiple R7 cells in flies with *sev* gain-of-function mutations. Furthermore, the association of Drk with an activated form of Sev has been demonstrated by both *in vitro* binding and co-immunoprecipitation experiments. This interaction is blocked by mutations that affect conserved residues of

the Drk SH2 domain involved in phosphotyrosine binding (Olivier *et al.*, 1993).

Here we present the results of a combined biochemical and genetic analysis of the sequences required for the functional interactions between Sev and Drk and between Drk and Sos. The Sev signalling pathway is particularly well suited for this purpose because mutations in *sev* or in *drk* can be analysed *in vivo*. Using a genetically sensitized system, even small changes in the efficiency of signal transduction can be detected. We show that a single tyrosine residue in the C-terminal tail of the Sev protein plays a central role in the binding of Drk and in mediating efficient signalling by the Sev receptor. Furthermore, we show that binding of Drk to Sos is mediated primarily by the N-terminal SH3 domain and that there is comparatively little contribution of the C-terminal SH3 domain to Drk function.

Results

Tyr 2546 is essential for binding of Drk to Sev RTK in vitro

In vitro studies have shown that the Drk SH2 domain preferentially binds to the phosphopeptide motif pYXNX (single amino acid code, where X can be any amino acid, Songyang *et al.*, 1994). Similar results have been obtained for the Sem-5 and Grb2 SH2 domains (Songyang *et al.*, 1993, 1994). This motif is found only once in the cytoplasmic domain of the Sev protein, suggesting that this site (Y2546ANE in the C-terminal tail of Sev; Basler and Hafen, 1988) may represent an autophosphorylation site that interacts with Drk. To test this hypothesis, we mutated the corresponding codon in the *sE-torso*^{4021}*–sev* transgene which encodes a constitutively active Sev kinase. We have previously shown that the fusion of the extracellular domain of the mutant Torso^{4021} RTK carrying an activating amino acid substitution in its extracellular domain (Sprenger and Nüsslein-Volhard, 1992) with the Sev kinase domain results in the strong constitutive activation of the Sev kinase (Dickson *et al.*, 1992). The transgene contains the *sev* enhancer and the *hsp70* heat-shock promoter. The *sev* enhancer controls expression of the transgene in the R7 equivalence group and hence permits analysis of the encoded protein in the Sev pathway. The *hsp70* heat-shock promoter allows the ubiquitous expression of the mutant protein in all cells of the fly providing sufficient material for biochemical analysis. Several independent transgenic lines were produced with this construct (*sE-torso*^{4021}*–sev*^{Y2546F}). An identical construct with the K2242M mutation in the ATP binding site of the Sev kinase domain was used as a negative control (*sE-torso*^{4021}*–sev*^{K2242M}, Dickson *et al.*, 1992).

To test the ability of the Torso^{4021}–sev^{Y2546F} protein to associate with Drk, we heat shocked flies carrying the *sE-torso*^{4021}*–sev*, the *sE-torso*^{4021}*–sev*^{Y2546F} or the *sE-torso*^{4021}*–sev* ^{K2242M} transgenes for 45 min at 37°C to induce ubiquitous expression of the transgenes and allowed the flies to recover at room temperature for 3 h. Protein extracts of these flies were then incubated with either anti-Sev or anti-Drk antisera, and the chimeric Sev proteins were identified in the anti-Sev and anti-Drk immunoprecipitates by immunoblotting with anti-Sev or anti-phosphotyrosine antibodies. Upon heat induction, all three

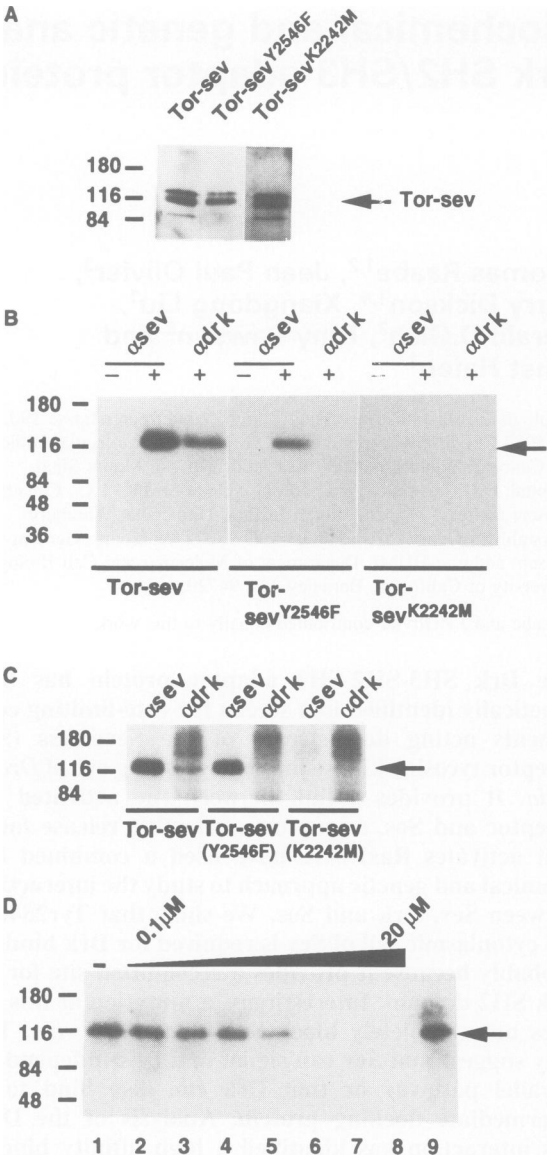

Fig. 1. Binding of Drk to Sev and Sev^{Y2546F} receptor tyrosine kinases (A and B). Adult flies harbouring the *sE-torso*^{4021}*–sev*, the mutant *sE-torso*^{4021}*–sev*^{Y2546F} or the kinase-defective *sE-torso*^{4021}*–sev*^{K2242M} transgenes were uninduced (–) or heat-shocked (+) and homogenized. (**A**) Lysates of heat-shocked flies were immunoprecipitated with anti-Sev sera and subjected to Western blot analysis with anti-Sev antibodies to show the expression level of wild-type and mutant chimeric Torso^{4021}–sev proteins. Lysates were immunoprecipitated with anti-Sev or anti-Drk antibodies, and the immune complexes were subjected to Western blot analysis with anti-phosphotyrosine antibody (**B**) or subjected to *in vitro* kinase reactions (**C**). (**D**) Phosphopeptide inhibition of Drk association with Torso^{4021}–sev. Lysates containing Torso^{4021}–sev were incubated with 2.5 μg of GST–Drk alone (lane 1) or in the presence of increasing concentrations (0.1, 0.2, 0.5, 1, 10 and 20 μM), lanes 2–8, of the phosphopeptide pY2546 (KQLpYANEGVSR). 20 μM of the phosphopeptide SSNpYMAPYDNY was used as a negative control, lane 9. After 90 min at 4°C, the beads were washed and associated Torso–sev was identified by Western blot analysis with anti-phosphotyrosine antibodies. The mobility of the Torso^{4021}–sev protein is indicated.

Torso^{4021}–sev chimeras accumulated to similar levels in the different transformant lines (Figure 1A). The Torso^{4021}–sev receptor could be detected in anti-Drk immune complexes, indicating that Torso^{4021}–sev is associated with Drk (Figure 1B). However, neither the Torso^{4021}–sev^{Y2546F},

nor the kinase-defective receptor were detectable in anti-Drk immunoprecipitates.

To test the ability of the different Torso^{4021}–sev proteins to autophosphorylate, *in vitro* kinase reactions were performed on anti-Sev and anti-Drk immune complexes obtained from heat-shocked flies (Figure 1C). Autophosphorylation of the Torso^{4021}–sev^{Y2546F} receptor, precipitated with anti-Sev antibodies, was reduced slightly compared with the Torso^{4021}–sev protein, while the Torso^{4021}–sev^{K2242M} mutant appeared incapable of autophosphorylation. This indicates that the Torso^{4021}–sev^{Y2546F} is active and that there are other major autophosphorylation sites in the Sev cytoplasmic domain. Consistent with the results discussed above, only Torso^{4021}–sev was detected in anti-Drk immunoprecipitates using the *in vitro* autokinase assay. Immunoblot analysis of the *in vitro* kinase reactions with anti-Sev sera revealed that approximately equal amounts of the different Torso^{4021}–sev chimeric proteins were made (data not shown).

To investigate whether autophosphorylation at Tyr2546 might create a direct binding site for the Drk SH2 domain, a synthetic phosphopeptide of 11 amino acids (KQLpYANEGVSR) was synthesized that corresponds to this region of the Sev tail. This pY2546 phosphopeptide was used in a competition assay involving the binding of the Torso^{4021}–sev receptor to Drk. The pY2546 phosphopeptide, at varying concentrations, was incubated along with a GST–Drk fusion protein in the presence of protein lysates from heat-shocked *sE-torso^{4021}–sev* flies. Associated Torso^{4021}–sev protein was identified by immunoblotting with anti-phosphotyrosine antibody. The pY2546 phosphopeptide inhibited the interaction of GST–Drk with Torso^{4021}–sev with an IC_{50} of ~500 nM (Figure 1D). In contrast, a phosphopeptide (SSNpYMAPYDNY), corresponding to the Tyr771 autophosphorylation site on the β receptor for platelet-derived growth factor, at a concentration of 20 μM had no effect on the association of GST–Drk with the activated Sev receptor. These results are consistent with a model in which the association of Sev with Drk is dependent on receptor autophosphorylation, primarily at Tyr2546.

A mutation in Tyr2546 of Sev reduces but does not eliminate Sev function in vivo

Given the dramatic effect of the Y2546F mutation on Drk binding *in vitro*, we wanted to know whether this mutation interferes with signalling from the Sev receptor *in vivo*. Previously it was shown that the constitutive activation of the Sev receptor under the control of the *sev* enhancer results in the recruitment of additional R7 cells (Basler *et al.*, 1991; Dickson *et al.*, 1992). The number of additional R7 cells is a sensitive measure for the signalling efficiency from the receptor. Flies that lack endogenous *sev* gene function due to the *sev^{d2}* null allele but carry one copy of the *sE-torso^{4021}–sev* transgene have rough eyes with multiple R7 cells per ommatidium (compare Figure 2A, D and B, E). Flies carrying one copy of the mutant *sE-torso^{4021}–sev^{Y2546F}* construct possess smooth eyes (Figure 2C, F). To quantify the extent of the suppression caused by the Y2546F mutation, we counted the number of R7 photoreceptor cells per ommatidium in several independent transformant lines of the *sE-torso^{4021}–sev* and the *sE-torso^{4021}–sev^{Y2546F}* construct. Compared with the transformants carrying the *sE-torso^{4021}–sev* transgene, which on average possess 3.8 ± 1.0 (n=128) R7 cells per ommatidium, the number of R7 cells is reduced to an average of 0.3 ± 0.3 (n=241) R7 cells per ommatidium in the three *sE-torso^{4021}–sev^{Y2546F}* transformants lines analysed. Interestingly, the *sev^{d2}*, *sE-torso^{4021}–sev^{Y2546F}* flies still contain some ommatidia with one R7 cell (Figure 2F). Furthermore, flies carrying two copies of the *sE-torso^{4021}–sev^{Y2546F}* construct possess rough eyes containing ommatidia with multiple R7 cells similar to flies carrying a single copy of the unmutated *sE-torso^{4021}–sev* transgene (data not shown). This indicates that the Torso^{4021}–sev^{Y2546F} protein still retains residual activity despite the lack of the putative Drk binding site.

Since the rough eye phenotype of *sE-torso^{4021}–sev* is caused by the transformation of the cone cell precursors into R7 cells and not by the recruitment of the R7 precursor itself, we wanted to test whether the same mutation also prevents the specification of the R7 cell proper. To test the effect of the Y2546F mutation in a sensitized system for the R7 cell, we introduced the Y2546F mutation into a transgene that encodes a partially functional Sev protein carrying a mutation in the extracellular domain (*sE-sev^{351}*, B.D. and E.H., unpublished). In a *sev* mutant background, flies homozygous for the transgene possess on average 0.81 ± 0.06 (n=354) R7 cells per ommatidium. In contrast, in flies carrying the identical construct with the Y2546F mutation, the number of R7 cells is reduced to 0.03 ± 0.04 (n=365). Therefore, this mutation reduces the efficiency of signalling from Sev in all cells that can adopt an R7 cell fate.

The N-terminal SH3 domain of Drk is required for binding to the proline-rich C-terminus of Sos

Drk has been previously shown to associate *in vitro* with the C-terminal proline-rich tail of Sos (Olivier *et al.*, 1993; Simon *et al.*, 1993), and similar results have been obtained for the mammalian proteins Grb2 and mSos1 (Egan *et al.*, 1993; Li *et al.*, 1993, Rozakis-Adcock *et al.*, 1993). To test whether both SH3 domains of Drk contribute equally to binding of Sos, we mutated the highly conserved tryptophan residue (W36A) in the N-terminal SH3 domain or the analogous residue (W189A) in the C-terminal SH3 domain of Drk to alanine. Purified soluble wild-type Drk, mutant Drk^{W36A} or Drk^{W189A} proteins were tested for their capacity to bind Sos fusion proteins immobilized on agarose beads. For this analysis, residues 1225–1405 from the C-terminal region of the Sos tail, including three proline-rich motifs that are potential SH3 binding sites, were expressed as a GST fusion protein (GST–Sos tail). A distinct GST–Sos fusion protein (GST–SosN) containing residues 505–692 from the amino-terminal region of Sos was used as a control. Binding of wild-type or mutant Drk to Sos was monitored by immunoblotting with anti-Drk antibodies. Wild-type Drk bound specifically to the Sos tail, but not to GST–SosN. Compared with wild-type Drk, the W36A and W189A mutants were reduced in their association with the Sos tail, on average by 85% and 53%, respectively (Figure 3A). Similar results were obtained using a filter binding assay, in which the GST–Sos tail was immobilized on a filter and probed with wild-type or mutant Drk proteins. In this assay, the W36A mutant Drk protein failed to associate detectably with the

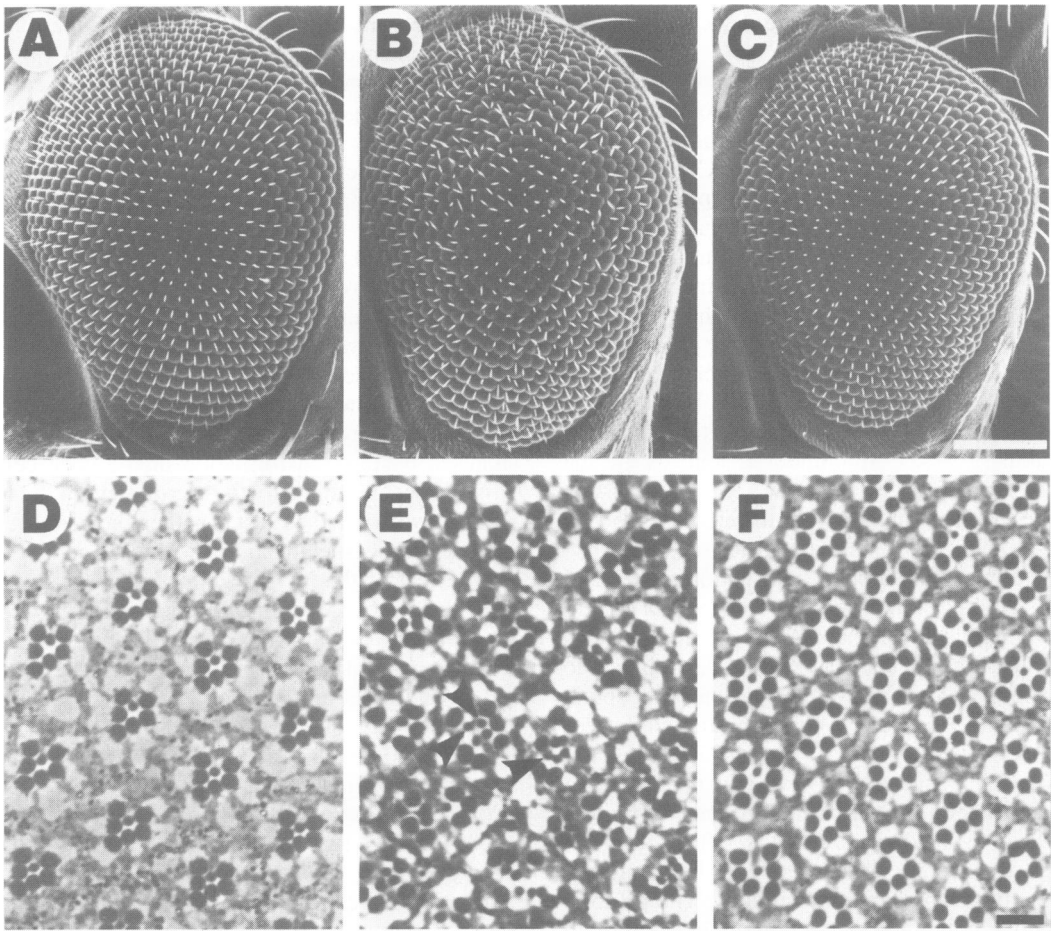

Fig. 2. The *sev* gain-of-function phenotype of *sE-torso*^{4021}*–sev* is suppressed by a mutation in the codon for the potential Drk binding site (Y2546). Scanning electron micrographs and histological sections of wild-type (**A, D**), *sE-torso*^{4021}*–sev* (**B, E**) and *sE-torso*^{4021}*–sev*^{Y2546F} (**C, F**) eyes. The *sE-torso*^{4021}*–sev* flies possess rough eyes owing to the recruitment of multiple R7 cells (arrowheads in E). The number of R7 cells is greatly reduced in flies expressing the *sE-torso*^{4021}*–sev*^{Y2546F} construct (F). The experiments were carried out in a w^{1118}, sev^{d2} background. The scale bars represent 100 μm in (C) and 10 μm in (F).

Sos tail, whereas W189A was reduced in its Sos binding (data not shown). These results suggest that the more N-terminal of the two Drk SH3 domains is primarily responsible for the association of Drk with Sos.

We wanted to exclude the possibility that the observed binding of the mutant Drk proteins was in part due to residual activity of the SH3 domain carrying the Trp to Ala mutation. We therefore constructed two mutant Drk proteins that entirely lack either the N-terminal SH3 domain [SH2-SH3(C)] or the C-terminal SH3 domain [SH3(N)-SH2]. These truncated Drk proteins were produced in bacteria and analysed for their ability to bind the Sos tail in a filter binding assay. Immobilized GST protein alone was unable to bind to the wild-type Drk protein (Figure 3B, lane 1). The mutant Drk protein lacking the C-terminal SH3 domain bound *in vitro* to the Sos tail with an efficiency approaching that of full-length wild-type Drk protein (Figure 3B, lanes 2 and 3). In contrast, the mutant Drk protein lacking the N-terminal SH3 domain bound only very weakly to the Sos tail (Figure 3B, lane 4). These results suggest that the N-terminal SH3 domain is both necessary and sufficient for efficient association with Sos *in vitro*.

Mutations in the SH3 domains of Drk impair Sev signal transduction to a different degree

The W36A and W189A substitutions in the Drk SH3 domains have distinct effects on Sos binding, suggesting that the N- and C-terminal SH3 domains of Drk may not be of equal functional importance. To test this hypothesis *in vivo*, we placed the wild-type and mutant *drk* genes under the control of the *sev* enhancer and the *hsp70* heat-shock promoter and generated transgenic flies (*sE-drk*; *sE-drk*^{W36A}; *sE-drk*^{W189A}). Protein expression of the different transgenes was verified in a *drk* null mutant background. Animals homozygous for the $drk^{\Delta P24}$ allele die as late third instar larvae and show no detectable Drk protein on Western blots (Figure 4, lane 2). $drk^{\Delta P24}$ third instar larvae carrying the different *drk* transgenes were heat shocked to induce transgene expression, and analysed for the presence of Drk protein by Western blotting using an anti-Drk antiserum. Upon heat induction, similar levels of Drk protein accumulated in the transgenic strains expressing either the wild-type (Figure 4, lane 3) or the mutant proteins (Figure 4, lanes 4 and 5). Both the wild-type and the mutant Drk proteins appear to be very stable. We did not observe a substantial reduction in the amount of

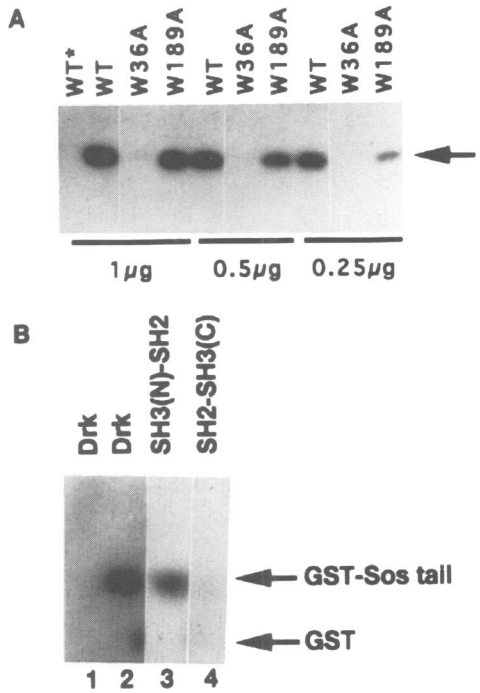

Fig. 3. Substitution of conserved residues in the SH3 domains of Drk reduce the affinity for Sos. (**A**) GST–SosN and GST–Sos tail fusion protein were expressed in bacteria and purified with glutathione–agarose beads. Immobilized GST–SosN and GST–Sos tail were mixed with 1, 0.5 or 0.25 μg of purified wild-type Drk, Drk^{W36A} or Drk^{W189A}, incubated at 4°C for 90 min and washed. Complexes were analysed by immunoblotting with anti-Drk antibodies. WT* designates the negative control where GST–SosN was mixed with 1 μg of wild-type Drk. The arrow indicates the mobility of Drk. (**B**) The N-terminal SH3 domain of Drk is important for binding to the proline-rich Sos tail. Purified GST (lane 1) and GST–Sos tail fusion proteins (lanes 2–4) were fractionated by SDS–PAGE and transferred to nitrocellulose. The filter was then incubated with purified wild-type Drk (lane 1 and 2), or deletion mutants of Drk, SH3(N)-SH2 (lane 3) or SH2-SH3(C) (lane 4) proteins. Binding of Drk and Drk mutants to the immobilized GST–Sos fusion proteins was detected by immunoblotting with anti-Drk antibodies. The Drk antiserum recognizes the various mutant forms of Drk with comparable efficiency to the wild-type protein. An arrow indicates the position of GST alone (lane 1) and GST–Sos tail (lanes 2–4).

protein even 16 h after the induction by heat shock (data not shown).

To assay the function of the Drk^{W36A} and Drk^{W189A} mutant proteins in the Sev pathway, we tested their ability to substitute for the function of the wild-type Drk protein in a *sE-torso*^{4021}*–sev*; *drk*^{R1}/+ background. The results of this analysis are shown in Figure 5 and their quantification is shown in Figure 5G. We have previously shown that the removal of one copy of the endogenous *drk* gene suppresses the rough eye phenotype caused by a constitutively activated Sev RTK (Olivier *et al.*, 1993). In *sE-torso*^{4021}*–sev* flies, 99% of the ommatidia contain multiple R7 cells (Figure 5A). Removal of one copy of *drk* reduces the fraction of ommatidia with multiple R7 cells to 33% (*sE-torso*^{4021}*–sev*; *drk*^{R1}/+, Figure 5B). Expression of the wild-type *drk* transgene in *sE-torso*^{4021}*–sev*; *drk*^{R1}/+; *sE-drk* flies fully complements the suppression caused by the *drk*^{R1} mutation and restores this value to 95% (Figure 5C). In contrast, only 22% of the ommatidia possess multiple R7 cells in flies carrying the transgene that encodes a Drk

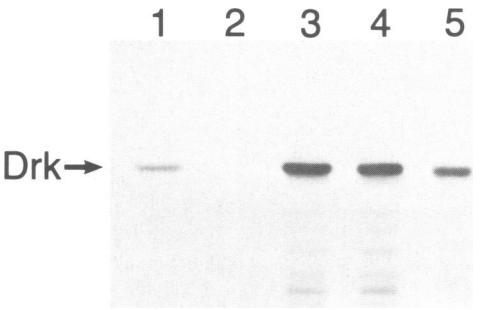

Fig. 4. Expression of *drk* under the control of the *hsp70* promoter. Third instar larvae of the following genotype were subjected to a 1 h heat shock at 37°C: *w*^{1118} (1); *w*^{1118}; *drk*$^{\Delta P24}$/*drk*$^{\Delta P24}$ (2); *w*^{1118}; *drk*$^{\Delta P24}$/*drk*$^{\Delta P24}$; *sE-drk*/ *sE-drk* (3); *w*^{1118}; *drk*$^{\Delta P24}$/*drk*$^{\Delta P24}$; *sE-drk*^{W36A}/*sE-drk*^{W36A} (4); *w*^{1118}; *drk*$^{\Delta P24}$/*drk*$^{\Delta P24}$; *sE-drk*^{W189A}/*sE-drk*^{W189A} (5). After 4 h at 25°C lysates were prepared and Drk expression was monitored by Western blotting with an anti-Drk antiserum. Compared with wild-type larvae, high levels of Drk protein are detected in all transgenic lines.

protein with a mutant N-terminal SH3 domain (Figure 5D). This suggests that this protein is unable to provide normal Drk function. However, 78% of all ommatidia in *sE-torso*^{4021}*–sev; drk*^{R1}/+ flies carrying the *sE-Drk*^{W189A} construct have more than one R7 cell (Figure 5E), suggesting the mutation in the C-terminal SH3 domain has comparatively little effect on Drk function in the Sev pathway. These results reflect the *in vitro* binding properties. Since even a Drk protein lacking the C-terminal SH3 domain was able to bind to the Sos tail we wanted to verify that this protein was functional *in vivo*. In flies carrying the *sE-drk*$^{SH3(N)\text{-}SH2}$ transgene, 76% of the ommatidia contain multiple R7 cells (Figure 5F). Therefore it appears that Drk can function in the Sev pathway even in the complete absence of its C-terminal SH3 domain.

Drk is also required in other developmental pathways (Simon *et al.*, 1991; Doyle and Bishop, 1993; Diaz-Benjumea and Hafen, 1994). The recessive lethality associated with *drk* mutations is fully rescued by the repeated ubiquitous induction of a wild-type *drk* cDNA under the control of the *hsp70* promoter (Olivier *et al.*, 1993). In contrast, neither the *drk*^{W36A} nor the *drk*$^{SH3(N)\text{-}SH2}$ were able to rescue *drk* mutant flies. Only the *drk*^{W189A} construct partially rescued *drk* homozygous mutant flies (20% of the rescue ability of the wild-type *drk* transgene). As in signalling downstream of the Sev RTK, the N-terminal SH3 domain appears to be essential for Drk function in other pathways. However, the fact that the Drk protein lacking the C-terminal SH3 domain was unable to rescue the lethality suggests that the C-terminal SH3 domain has a more important function in these other signalling pathways.

Drk binds to two proline-rich motifs in the C-terminal tail of Sos

The C-terminal region of Sos contains three central proline-rich motifs designated P1, P2 and P3, that might represent SH3 binding sites (see Figure 6A). To test for a direct interaction of Drk with these regions of Sos, GST fusion proteins containing one or more of these proline-rich

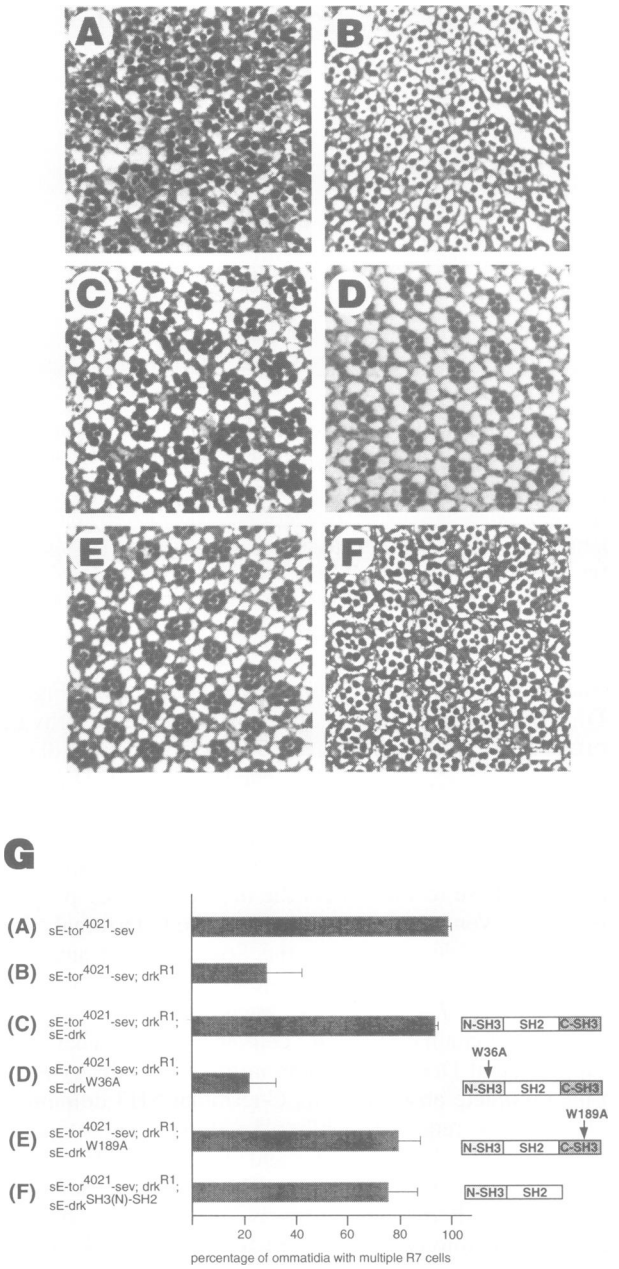

Fig. 5. Functional analysis of the SH3 domains in Drk *in vivo*. Histological sections of the eyes of flies with the following genotypes are shown: *sE-torso*^{4021}*–sev* (**A**), *sE-torso*^{4021}*–sev, drk*^{R1}*/+* (**B**), *sE-torso*^{4021}*–sev, drk*^{R1}*/+; sE-drk /+* (**C**), *sE-torso*^{4021}*–sev, drk*^{R1}*/+; sE-drk*^{W36A}*/+* (**D**), *sE-torso*^{4021}*–sev, drk*^{R1}*/+; sE-drk*^{W189A}*/+* (**E**), *sE-torso*^{4021}*–sev, drk*^{R1}*/+; sE-Drk*$^{SH3(N)\text{-}SH2}$*/+* (**F**). The multiple R7 cell phenotype of *sE-torso*^{4021}*–sev* (A) is suppressed in flies heterozygous for *drk*^{R1} (B). This suppression is reverted by expression of the wild-type *drk* cDNA under the control of the *sev* enhancer (*sE*) (C). A single amino acid exchange in the N-terminal SH3 domain (W36A) of Drk prevents the rescue ability (D), whereas the corresponding substitution in or the complete removal of the C-terminal SH3 domain have little effect on Drk function (E and F). A quantification of the results presented in A–F are shown in (**G**). The bars represent the percentage of ommatidia containing more than one R7 cell. For each construct, two heads of two independent transformant lines were sectioned and the ommatidia counted. The scale bar in (F) represents 10 μm.

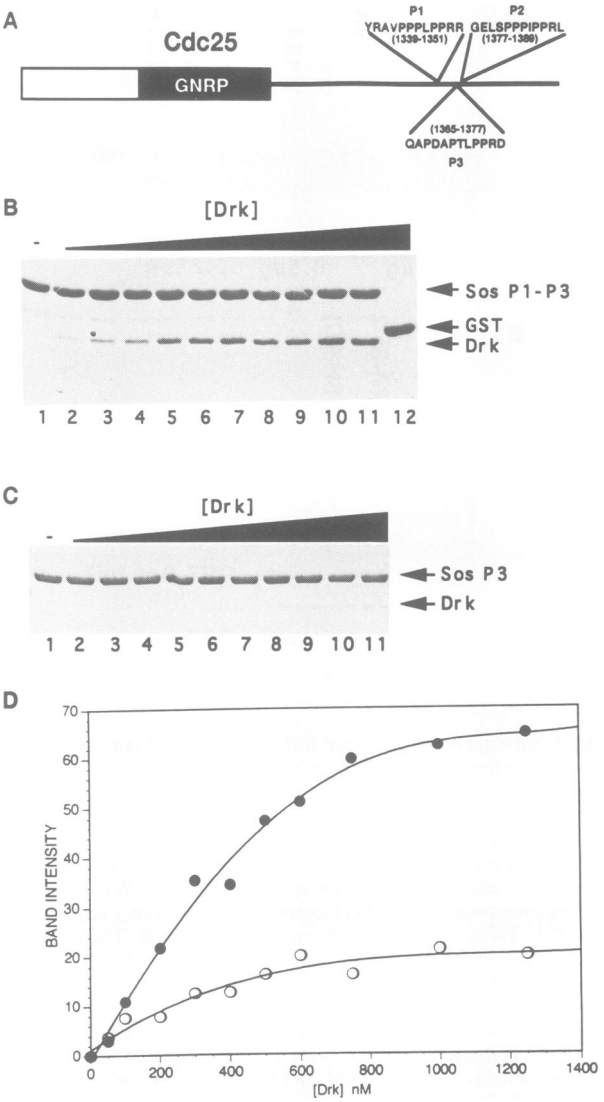

Fig. 6. Concentration dependence of Drk binding to truncated GST–Sos fusion proteins. (**A**) Schematic diagram illustrating the structural domains of Sos. The N-terminus (white box) of Sos contains a Dbl homology domain and a PH domain. The Cdc25 domain is likely to function as the catalytic domain with guanine nucleotide exchange activity. The C-terminus is proline-rich with three motifs labelled P1–P3 that resemble potential SH3 binding sites. Purified Drk protein, at concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 750, 1000 and 1250 μM (lanes 1–11) was incubated with a volume of bacterial lysate containing ~300 nM of (**B**) GST–Sos P1–P3 or GST alone (lane 12) or (**C**) GST–Sos P3 in 1 ml PLC lysis buffer for 2 h at 4°C. Glutathione–agarose beads were added to the mixture, and the incubation continued for 20 min. The beads were washed, and bound proteins were resolved by 15% SDS–PAGE and detected by Coomassie Blue staining. (**D**) Graph of Drk band intensity, as determined by optical densitometry, as a function of the initial Drk concentration. Filled and open circles represent Drk binding to GST–Sos P1–P3 and GST–Sos P3, respectively.

sequences were prepared. One of these fusion proteins (GST–Sos P1–P3) contains the entire region spanning the P1–P3 sites (residues 1313–1387), while others contain individual proline-rich motifs. For example, GST–Sos P3 contains residues 1353–1387, spanning the P3 site. These GST–Sos fusion proteins were immobilized on glutathione–Sepharose beads and tested for their ability to bind soluble Drk protein. Whereas no Drk was precipitated in control experiments using GST alone, each of the GST–Sos fusion proteins bound Drk. Furthermore, when tested at approximately equivalent molar concentrations, the

GST–Sos fusion proteins containing multiple proline-rich sequences appeared to bind more Drk than fusions with individual proline-rich motifs. This is illustrated in Figure 6, where concentrations of purified Drk ranging from 50 to 1250 nM were tested for binding to ~300 nM of GST–Sos P1–P3 fusion protein (Figure 6B). This is compared with the result obtained using 300 nM of a fusion protein containing a single motif, GST–Sos P3 (Figure 6C), and to GST alone. Optical densitometry of the Coomassie Blue-stained Drk band revealed that almost four times more Drk was bound to the construct containing multiple proline-rich motifs (Figure 6D).

To further delineate the likely contact points on Sos for the Drk SH3 domain, synthetic peptides corresponding to three proline-rich motifs within the region of Sos shown to interact directly with Drk were synthesized, and employed in competition experiments. In these studies, the peptides, at concentrations from 70 to 700 nM, were incubated in a solution containing 600 nM Drk and 300 nM GST–Sos P1–P3 fusion protein for 90 min at 4°C. Following this period, excess GST beads were added and the mixture allowed to incubate for a further 30 min After washing, the beads were analysed for the amount of Drk present and compared with a control where no peptide was added. As shown in Figure 7A, two of the peptides displayed inhibitory properties. Peptide P1, sequence YRAVPPPLPPRR, showed the strongest inhibition, IC_{50}=50 μM; while P2, sequence GELSPPPIPPRL, was poorer, IC_{50} = 280 μM (Figure 7B). The third peptide, P3, sequence GAPDAPTLPPRD, showed little inhibition.

Taken together, these results indicate that the N-terminal Drk SH3 domain is principally responsible for the binding of Drk to Sos. Furthermore, analysis of potential SH3 binding sites has defined specific proline-rich motifs in the Sos tail that can associate with Drk *in vitro*. The highest affinity site is represented by the peptide YRAVPPPLPPRR. However, Sos polypeptides containing multiple proline-rich motifs apparently bound Drk more efficiently than proteins with single proline-rich motifs.

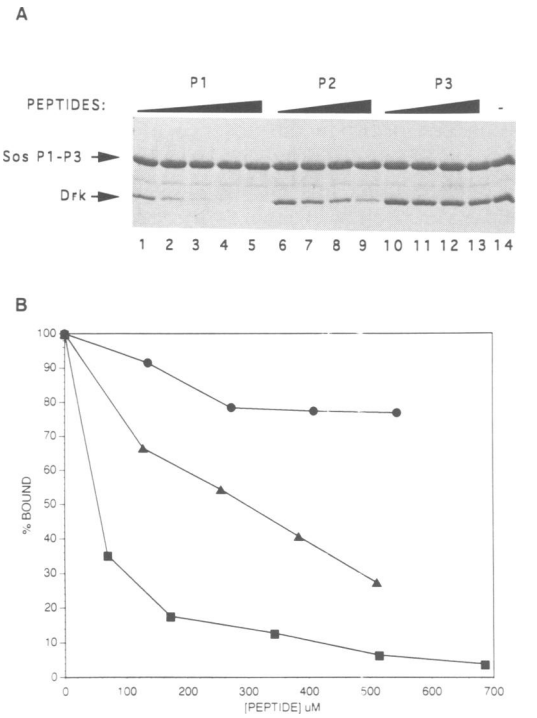

Fig. 7. (**A**) Peptide competition of Drk binding to truncated GST–Sos P1–P3 fusion protein. Approximately 600 nM of purified Drk protein were incubated with bacterial lysates containing 300 nM GST–Sos P1–P3 fusion protein in the presence of proline-rich peptides P1 (YRAVPPPLPPRR), P2 (GELSPPPIPPRL) and P3 (GAPDAPTLPPRD), at concentrations of 70, 172, 343, 515 and 686 μM, lanes 1–5, 128, 256, 384 and 512 μM, lanes 6–9 and 136, 273, 409 and 545 μM, lanes 10–13. No peptide was added as a control in lane 14. Following a 90 min incubation period at 4°C, glutathione–agarose beads were added and the incubation continued for 30 min. The beads were washed, and bound proteins resolved by 15% SDS–PAGE and detected by Coomassie Blue staining. (**B**) The magnitude of peptide inhibition, monitored relative to a control with no peptide added, was determined from the intensity of the Drk band estimated by optical densitometry, plotted in a graph as a function of peptide concentration. ■ = peptide P1; ▲ = peptide P2; ● = peptide P3.

Discussion

In this study we have combined biochemical and genetic assays to investigate the functional interactions between the Sev receptor tyrosine kinase, the SH2/SH3 adaptor Drk and the guanine nucleotide exchange factor Sos.

Sev–Drk interactions

The only sequence in the Sev cytoplasmic domain that fits the YXNX binding consensus for Sem-5-type SH2 domains (Songyang *et al.*, 1993, 1994) is the sequence Y(2546)ANE in the C-terminal tail of the Sev receptor. Substitution of Tyr2546 with Phe results in a lower degree of Sev autophosphorylation (Figure 1), and loss of binding to Drk. Furthermore, the binding of Drk to Sev can be competed with a phosphopeptide corresponding to the C-terminal tail of Sev containing Tyr2546. These results suggest that Tyr2546 is an autophosphorylation site required for binding of the Drk SH2 domain. Since these experiments have been carried out in fly extracts we cannot rule out that the binding of Drk to Sev is indirect via another protein. The functional significance of Tyr2546 is supported by our *in vivo* experiments. Introducing the Y2546F substitution into a constitutively active form of the Sev receptor or a partially functional receptor significantly impairs the specification of the R7 cell fate in the R7 equivalence group (Figure 2).

Given the dramatic effect of the Y2546F mutation on the binding of activated Sev to Drk, it is surprising, however, that this mutation does not completely abolish the ability of Sev to specify R7 cells. Since these experiments have been carried out in a *sev* null mutant background, the only source of Sev protein is the mutant $Torso^{4021}$–sev^{Y2546F} or the partially functional Sev^{351} receptor containing the Y2546F substitution, respectively. In each case R7 cells can still form, albeit at a lower frequency. Furthermore, rescue experiments with a genomic DNA fragment encompassing the entire *sev* gene in which this Y2546F mutation had been introduced showed that this mutant *sev* gene could completely rescue the *sev* mutant phenotype (K.Basler, C.Bierkamp and E.H., unpublished data). This indicates that the levels of Sev activity in the R7 precursor cell containing a wild-type Sev receptor are sufficient to specify R7 cell development even in the absence of the Y2546 autophosphorylation site. In either a sev^{351} or a *sE-torso*^{4021}–*sev* background,

however, the Sev activity is at a critical threshold where the Y2546F mutation has a dramatic effect on the efficiency of R7 cell specification.

There are at least three possibilities to explain how Sev signalling can occur in the absence of the putative Drk binding site. (i) Drk is able to bind the phosphorylated Sev receptor at other low affinity sites. (ii) Drk binds Sev indirectly via another protein. (iii) Sev can signal in a Drk-independent parallel pathway. The absence of additional SH2 binding consensus sequences for the Drk/Grb2/Sem-5-type SH2 domains in the cytoplasmic domain of Sev, together with the complete failure to immunoprecipitate the mutant Sev protein with the anti-Drk antiserum, makes the first explanation unlikely. From biochemical studies on vertebrate RTKs there are precedents for the second and the third possibilities. It has been shown that the tyrosine phosphorylation of proteins such as Shc, IRS-1 and Syp at YXNX motifs can create binding sites for Grb2 (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Baltensperger *et al.*, 1993; Feng *et al.*, 1993; Pronk *et al.*, 1993; Skolnik *et al.*, 1993a,b; Vogel *et al.*, 1993, Li *et al.*, 1994). While both Shc and Syp have SH2 domains, Shc can also bind to activated receptors in an SH2 domain-independent way through its PTB domain (Blaikie *et al.*, 1994; Kavanaugh and Williams, 1994). IRS-1 lacks an SH2 domain, but interacts with the juxtamembrane region of the insulin receptor. The Syp homologue Corkscrew (Csw) in *Drosophila* may similarly act as an intermediate between Sev and Drk. Genetic evidence for a role of Csw in the Sev pathway has been obtained. Mutations in *csw* have been shown to impair signalling in the Torso and Sev pathways (Simon *et al.*, 1991; Perkins *et al.*, 1992; M.Simon personal communication). It is interesting to note that the Grb2 binding site, pYTNI in Syp (Bennett *et al.*, 1994), is perfectly conserved in Csw. There is, however, no direct evidence yet that Csw interacts with, or is a substrate for, the Sev receptor.

Evidence of the third possibility that RTK signalling occurs via a parallel Drk-independent parallel pathway, has also been obtained from vertebrates. It has been shown that activated RTKs can associate with and phosphorylate a number of SH2 domain-containing proteins, several of which can potentially activate the Ras pathway (Koch *et al.* 1991; Pawson and Schlessinger, 1993; Valius and Kazlauskas, 1993). For the colony-stimulating factor 1 (CSF-1) receptor it has been shown that only the combined mutation of Grb2 and phosphoinositol-3 (PI-3) kinase binding sites completely abrogates signal transduction, whereas mutation of either binding site alone reduced the ability to change morphology or growth rate in response to CSF-1 stimulation (van der Geer and Hunter, 1993). So far, there is no evidence for an involvement of PI-3 kinase in Sev-mediated signal transduction in *Drosophila*. The existence of a Drk-independent pathway is suggested, however, by the analysis of *drk* homozygous mutant cell clones in the eye or in the adult cuticle. The phenotypes of the strongest Drk alleles are less severe than those observed in clones of other mutations in the pathway, such as Raf, Ras1 or the corresponding receptors (Simon *et al.*, 1991; Diaz-Benjumea and Hafen, 1994). Although a residual Drk function in the alleles tested could explain the different strength in phenotypes, we think this is unlikely since both alleles tested result in the substitution of conserved amino acids in the SH2 domain which completely abolish SH2 binding *in vitro* (Olivier *et al.*, 1993). Furthermore, the Drk alleles tested exhibit the same degree of suppression of *sE-torso*^{4021}*–sev* as the *drk* null allele, *drk*$^{\Delta P24}$. We therefore consider it quite likely that a parallel, Drk-independent pathway leading to the activation of Ras1 and Raf exists. It is possible that mutations in genes coding for components in this pathway have not been identified because these components are not rate limiting for Sev signalling in the presence of Drk.

The interaction between Drk and Sos is mediated primarily via the N-terminal SH3 domain

In vitro binding studies indicated that a mutant Drk protein carrying a substitution of a conserved Trp (W189) to Ala in the C-terminal SH3 domain bound to Sos peptides with similar efficiency to the wild-type protein. W36 and W189 in Drk are residues that are invariably conserved in all known SH3 domains and are part of the peptide binding pocket (Koyama *et al.*, 1993). Furthermore, a truncated Drk protein that completely lacks the C-terminal SH3 domain is still able to bind to Sos, albeit with reduced efficiency. The results obtained *in vitro* correlate well with the results of our *in vivo* analysis of the Drk SH3 domains. Constructs encoding either the Drk^{W189A} protein or a Drk protein completely lacking the C-terminal SH3 domain were able to largely rescue the dominant suppression of the rough eye phenotype of *sE-torso*^{4021}*–sev* by *drk* null mutations. However, Drk with the substitution of the analogous residue in the N-terminal SH3 domain failed to bind efficiently to the Sos tail and was unable to rescue the suppression by *drk* of *sE-torso*^{4021}*–sev*. Therefore, the N-terminal SH3 domain is necessary and sufficient for efficient binding of Drk to Sos *in vitro*, and for efficient signal transduction from the Sev kinase *in vivo*.

Recent structural analysis has shown that the N- and C-terminal SH3 domains of Sem-5 and Grb2 bind to proline-rich peptides in the opposite orientation (i.e. C- to N-terminal) compared with previously observed SH3 complexes (Feng *et al.*, 1994; Lim *et al.*, 1994; Wittekind *et al.*, 1994) These Sem-5/Grb2 binding peptides contain the sequence NH2 X-P-p-X-P-p-R COOH where X is an aliphatic residue, p is a scaffolding residue (often a proline) that maintains the polyproline type II helix, and the C-terminal arginine forms a salt bridge with an acidic SH3 domain residue. Additional C-terminal arginines appear to increase the binding affinity (Feng *et al.*, 1994). The high affinity binding site for Drk on *Drosophila* Sos, represented by the P1 peptide YRAVPPPLPPRR, conforms to this consensus. The aliphatic-proline pairs (P–P; L–P) probably fit into the hydrophobic binding pockets on the Drk N-terminal SH3 domain, while the underlined arginine potentially makes a salt bridge with Glu16 (Lim *et al.*, 1994; Wittekind *et al.*, 1994). Our data suggest that the N-terminal Drk SH3 domain is primarily responsible for binding to Sos, and may contact the YRAVPPPLPPRR (P1) site in the Sos tail.

The *in vitro* binding studies suggest that the C-terminal SH3 domain enhances the binding of the N-terminal SH3 domain to Sos. The wild-type form of Drk binds more strongly to the Sos tail than either Drk^{W189A} or the C-terminally truncated Drk. Similarly, the *drk* transgenes

with a mutation or a deletion in the C-terminal SH3 domain are somewhat less efficient in rescuing the *drk* function than the wild-type gene. It is therefore likely that the C-terminal SH3 domain of Drk strengthens the Drk–Sos interaction by interacting with a second proline-rich sequence, such as the P2 sequence, upon binding of the N-terminal SH3 domain to the P1 sequence. The C-terminal Drk SH3 domain might also play a role distinct from binding to Sos. It has also been shown that both SH3 domains of Grb2 interact with Dynamin, a GTPase that functions in endocytosis and synaptic transmission (Gout *et al.*, 1993; Scaife *et al.*, 1994). The C-terminal SH3 domain may similarly be involved in coupling Drk to other proteins that do not play a major role in Sev signalling. A more central role of the C-terminal SH3 domain of Drk in other signalling pathways is suggested by our observation that the Drk construct lacking the C-terminal SH3 domain was unable to rescue the lethality associated with *drk* mutations, whereas the same construct functioned to near normal levels in the Sev pathway.

Finally, this paper illustrates the importance of utilizing both *in vitro* assays and a sensitive *in vivo* analysis to decipher the functional importance of the individual domains of Drk. By applying both of these approaches we were able to identify a site in Sev required for association with Drk, to define contact sites between the Drk SH3 domains and Sos and to test the importance of these interactions *in vivo*.

Materials and methods

Genetics

Mutations in the *drk* gene were isolated in a genetic screen for second-site modifiers of the rough eye phenotype of the gain-of-function mutation Sev^{S11} which encodes a constitutively activated Sev protein (Basler *et al.*, 1991; Olivier *et al.*, 1993; T.R. and E.H., unpublished data). All experiments were performed in a sev^{d2} null mutant background (Basler *et al.*, 1991). Loss-of-function mutations in the *drk* gene lacking detectable levels of Drk protein were generated by mobilizing the P-element insertion drk^{P24} using a stable source of transposase (Robertson *et al.*, 1988). Based on the suppression of the $torso^{4021}$–*sev* rough eye phenotype, non-complementation of existing *drk* alleles and the absence of Drk protein on Western blots (see Results), $drk^{\Delta P24}$ was designated as a complete loss-of-function mutation. For the experiments described in Figure 5, transgenic lines carrying the different *drk* constructs were crossed to w^{1118}, sev^{d2} flies bearing the drk^{R1} mutation and the *sE*-$torso^{4021}$–*sev* transgene on the same chromosome. To test whether ubiquitous expression of the *drk* constructs could rescue the lethality of *drk* mutations, we crossed $drk^{\Delta P24}$/*Bc Gla* flies with $drk^{\Delta P24}$/*Bc Gla* flies homozygous for the *sE*-drk^{wt}, *sE*-drk^{W36A} or *sE*-drk^{W189A}, respectively. The temperature was shifted to 37°C for 60 min every 8 h throughout development. These mutant constructs behaved identically in independent rescue experiments with $drk^{R1}/drk^{E(sev)2B}$ flies (data not shown). The rescue ability of the $drk^{SH3(N)\text{-}SH2}$ construct lacking the C-terminal SH3 domain was tested in a cross of drk^{R1}/*Sco* flies with $drk^{E(sev)2B}$/*CyO*; $drk^{SH3(N)\text{-}SH2}$/+ using the same temperature treatment. Whereas the expected number of $drk^{R1}/drk^{E(sev)2B}$; *sE*-drk^{wt}/+ flies were obtained in the control experiment (79/305 flies carrying the *sE*-drk^{wt} construct), no $drk^{R1}/drk^{E(sev)2B}$; *sE*-$drk^{SH3(N)\text{-}SH2}$/+ flies survived (0/107).

Plasmid construction for germline transformations

The $torso^{4021}$–*sev* and $torso^{4021}$–sev^{K2242M} constructs are described in Dickson *et al.* (1992). The Y2546F substitution was introduced into $torso^{4021}$–*sev* by replacing a 1.1 kb *Bam*HI cDNA fragment with the corresponding fragment from a *sev* cDNA in which the Y2546 codon has been mutated by site-directed mutagenesis. The hypomorphic *sev* construct, sev^{351}, in which the codons for Y1485 and W1486 in the extracellular domain of Sev have both been replaced by alanine codons, was generated in the course of a structure–function analysis of the Sev extracellular domain (B.D. and E.H., unpublished data). The W36A and W189A Drk mutations were generated by site-directed mutagenesis of the *drk* cDNA using the oligonucleotides GGAAGACGATTCAAATGCATATCGCGCGGAGC and GCTCCGATGAGAACGCGTGGAACGGCGAG, respectively. For germline transformations a modified pW8 transformation vector was used (Klemenz *et al.*, 1987). All derivatives of the original $torso^{4021}$–*sev* and *drk* constructs are under the control of a single copy of the 1.2 *sev* enhancer fragment and the *hsp70* promoter (*sE*). Expression of the partially functional sev^{351} and $sev^{351\text{-}Y2546F}$ receptors is driven by a duplicated *sev* enhancer (Basler *et al.*, 1991). Transgenic lines were generated by injection of Quiagen purified plasmid DNA into w^{1118} sev^{d2} or w^{1118} embryos as described previously (Basler *et al.*, 1991). For all constructs, several independent transformant lines were established.

Scanning electron microscopy and histology

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

Western blotting and immunoprecipitation

For immunoprecipitations, adult flies were homogenized in PLC-LB [50 mM HEPES (pH 7.5), 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$ 1 mM EGTA, 100 mM NaF and 10 mM NaPPi] containing 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride. Lysates were clarified by centrifugation and incubated with 2 μg/ml affinity-purified anti-Drk or 1:200 dilution of anti-Sev, and protein A–Sepharose, for 90 min at 4°C. Immunoprecipitates were washed three times with 1 ml 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 8.25%, or 10% SDS–polyacrylamide gels, and transferred to nitrocellulose in a semi-dry blotting apparatus at 0.8 mA/cm^2 for 1 h. Filters were blocked for 1 h at room temperature in 5% non-fat 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 μg/ml affinity-purified anti-Drk, or 1:1000 dilution of anti-Sev. The anti-Drk antiserum was generated in a rabbit against a non-GST–Drk fusion protein (Olivier *et al.*, 1993). The anti-Sev antiserum (G24) was generated in a goat against a bacterially synthesized protein corresponding to cytoplasmic kinase domains of Sev. For anti-phosphotyrosine immunoblots, filters were blocked in 5% bovine serum albumin (BSA), 1% ovalbumin in TBS-T and probed with 1 μg/ml affinity-purified anti-phosphotyrosine antibodies as described previously (Letwin *et al.*, 1988). Anti-Drk and anti-Sev immunoblots were developed using either the ECL kit (Amersham), or [^{125}I]protein A (Amersham). The anti-phosphotyrosine immunoblots were processed using [^{125}I]protein A.

GST fusion proteins

Drk was amplified by polymerase chain reaction (PCR) and subcloned into the bacterial expression plasmid pGEX-KT (Pharmacia) as described previously (Olivier *et al.* 1993). The drk^{W36A} and drk^{W189A} mutants were produced by PCR and subcloned into pGEX-KT. The deletion mutants of Drk, SH3(N)-SH2 (amino acids 1–156) and SH2-SH3(C) (amino acids 60–211) were amplified by PCR and subcloned into pGEX-KT. Four regions of Sos were amplified by PCR and subcloned into the pGEX-KT vector; pGEX Sos-N encodes amino acids 505–692, pGEX Sos-tail amino acids 1225–1405, pGEX Sos P1-P3 amino acids 1313–1387, and pGEX Sos P3 amino acids 1353–1387. All plasmid constructs were sequenced using the Sequenase method (United States Biochemical Corporation) and transformed into *Escherichia coli* DH5α. Bacteria were grown to an OD_{600} of 0.6 and fusion proteins were induced at 30°C with 1 mM isopropyl-β-D-thiogalactosidase (IPTG) for 3 h. Cells were collected, sonicated in PBS-T^2 (PBS, 1% Triton X-100 and 1% Tween) and clarified by centrifugation. The supernatant was incubated with glutathione–agarose beads for 1 h at 4°C and washed three times with PBS-T^2.

In vitro binding studies

Adult flies were lysed in PLC lysis buffer at 10 flies per ml of buffer, the homogenate was cleared by centrifugation and incubated with immobilized GST or GST fusion proteins (2.5 μg) for 90 min at 4°C. All complexes were washed three times with HNTG, boiled for 5 min in SDS sample buffer, resolved by SDS–PAGE (8.25%) and transferred to nitrocellulose. Filters were blocked and probed with anti-phosphotyrosine 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 μCi of [γ-^{32}P]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× SDS sample buffer. A fraction of the kinase reaction was separated by SDS–PAGE and the gel was fixed, dried and exposed to film.

GST–SosN and GST–Sos tail fusion proteins were expressed in bacteria and purified with glutathione–agarose beads. Immobilized GST–SosN and GST–Sos tail were mixed with 1 μg, 0.5 μg or 0.25 μg of purified wild-type Drk, Drk^{W36A} or Drk^{W189A} and incubated for 90 min at 4°C. Complexes were washed three times with HNTG, boiled for 5 min in SDS sample buffer, resolved by SDS–PAGE (10%) and transferred to nitrocellulose. Filters were blocked and immunoblotted with anti-Drk antibodies and processed using [^{125}I]protein A. The amount of Drk was quantified using a PhosphorImager (Molecular Dynamics).

Purified GST, GST–SosN and GST–Sos tail bacterial fusion proteins, 1 μg of each, were resolved by 10% SDS–PAGE and were transferred to nitrocellulose. Filters were blocked in TBS containing 5% BSA, 1% ovalbumin and 0.1% SDS overnight at 4°C. These filters were probed using purified wild-type or mutant Drk protein from bacteria for 3 h at room temperature at 1 μg/ml and washed three times with TBS + 0.1% SDS. Complexes were detected by immunoblotting using affinity-purified anti-Drk antibodies in 5% BSA, 1% ovalbumin in TBS and processed with [^{125}I]protein A.

To determine the concentration dependence of Drk binding to truncated GST–Sos fusion proteins, purified Drk protein, at concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 750, 1000 and 1250 μM, was incubated with a volume of bacterial lysate containing ~300 nM of GST–Sos P1–P3, GST–Sos P3, or GST in 1 ml PLC lysis buffer for 2 h at 4°C. Forty μl of 50% (v/v) glutathione–agarose beads were added and the incubation continued for 20 min. Following three 1 ml PLC lysis buffer washes of the beads, bound proteins were resolved by 15% SDS–PAGE and detected by Coomassie Blue staining.

Peptide competition

The phosphopeptide pY2546 (KQLpYANEGVSR) was added to lysates containing Torso–sev, to final concentrations of 20, 10, 1, 0.5, 0.2 and 0.1 μM in the presence of 2.5 μg immobilized GST–Drk fusion protein and incubated for 90 min at 4°C. As a control, non-specific phosphopeptide pY771 (SSNpYMAPYDNY) was also used at 20 μM. All complexes were washed three times with HNTG, boiled for 5 min in SDS sample buffer, resolved by SDS–PAGE (8.25%) and transferred to nitrocellulose. Filters were blocked and probed with anti-phosphotyrosine antibodies as described above and quantified using a PhosphorImager (Molecular Dynamics).

Peptide competition of Drk binding to truncted GST–Sos P1–P3 fusion proteins

Approximately 600 nM of purified Drk protein was incubated with bacterial lysates containing 300 nM GST–Sos P1–P3 fusion protein in the presence of proline-rich peptides P1 (YRAVPPPLPPRR), P2 (GELSPPPIPPRL) and P3 (GAPDAPTLPPRD), at concentrations ranging from 70 to 700 μM (see Figure 7). Following a 90 min incubation period at 4°C, 40 ml of 50% (v/v) glutathione–agarose beads was added and the incubation continued for a further 30 min. The beads were washed with three 1 ml volumes of PLC lysis buffer, bound proteins resolved by 15% SDS–PAGE and detected by Coomassie Blue staining. The magnitude of peptide inhibition, monitored relative to a control with no peptide added, was determined from the intensity of the Drk band estimated by optical densitometry, plotted in a graph as a function of peptide concentration.

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