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DOS, a Novel Pleckstrin Homology Domain–Containing Protein Required for Signal Transduction between Sevenless and Ras1 in Drosophila

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Summary

The specification of the R7 photoreceptor cell in the developing eye of Drosophila is dependent upon activation of the Sevenless (SEV) receptor tyrosine kinase. By screening for mutations that suppress signaling via a constitutively activated SEV protein, we have identified a novel gene, *daughter of sevenless (dos)*. DOS is required not only for signal transduction via SEV but also in other receptor tyrosine kinase signaling pathways throughout development. The presence of an amino-terminally located pleckstrin homology domain and many potential tyrosine phosphorylation sites suggests that DOS functions as an adaptor protein able to interact with multiple signaling molecules. Our genetic analysis demonstrates that DOS functions upstream of Ras1 and defines a signaling pathway that is independent of direct binding of the DRK SH2/SH3 adaptor protein to the SEV receptor tyrosine kinase.

Introduction

Receptor tyrosine kinases (RTKs) play an essential role in the control of cell growth and differentiation during the development of multicellular organisms by mediating diverse cellular responses to extracellular signals. One of the immediate consequences of RTK stimulation is the activation of the Ras/MAP kinase pathway (McCormick, 1994; Marshall, 1994). The genetic dissection of RTK signaling pathways in Drosophila and Caenorhabditis elegans has revealed a set of cytoplasmic components that constitute an apparently linear signaling cascade from the receptor to Ras1 (reviewed by Duffy and Perrimon, 1994; Hafen et al., 1994; Simon, 1994; Stern and DeVore, 1994; Zipursky and Rubin, 1994). In this model, an SH2/SH3 (SRC homology 2/SRC homology 3) adaptor protein (DRK in Drosophila, Sem–5 in C. elegans, and Grb2 in vertebrates) binds with its SH2 domain to specific phosphotyrosine residues on the cytoplasmic domain of the activated receptor. With its SH3 domains, DRK interacts with the carboxy-terminal region of the guanine nucleotide exchange factor Son of Sevenless (SOS) and brings it in close proximity to Ras1. SOS activates Ras1 by catalyzing the GDP–GTP exchange (reviewed by McCormick, 1993; Pawson, 1995).

The simple linear model of RTK signal transduction has been questioned by the large bulk of biochemical evidence for a variety of proteins that are bound to activated RTKs (Kazlauskas, 1994; Schlessinger, 1994; Pawson, 1995). Furthermore, recent genetic evidence from the study of RTK signaling pathways in Drosophila also provides evidence for the activation of more than one signaling pathway in response to receptor stimulation. Activation of the Torso RTK during early embryogenesis can activate Raf, the downstream effector of Ras1, independent of Ras1 function (Hou et al., 1995). Local activation of Torso at the blastoderm stage is required for specification of the head and tail region of the larva. The RNA for receptor and signaling components is placed into the egg during oogenesis (reviewed by Duffy and Perrimon, 1994). Removal of DRK, SOS, or even Ras1 function during oogenesis results in a less severe phenotype than removal of Torso or Raf (Hou et al., 1995). Similarly, removal of DRK and SOS function in clones of cells in the developing wing results in a less severe effect on epidermal growth factor (EGF) receptor–mediated differentiation of veins and control of cell size than removal of the receptor itself (Diaz–Benjumea and Hafen, 1994). In the Sevenless (SEV) RTK pathway (Dickson, 1995; Hafen et al., 1994; Simon, 1994; Zipursky and Rubin, 1994), which is required for specification of cell fate in the developing eye, the effects of complete loss-of-function mutations of signaling components cannot be studied directly, since all the cytoplasmic components are also required for cell proliferation. However, analysis of the function of a SEV mutant receptor lacking the DRK binding site indicates that SEV signaling can occur, albeit at reduced efficiency, in the absence of direct binding of DRK to SEV (Raabe et al., 1995). Furthermore, the tyrosine phosphatase Corkscrew (CSW) has been shown to function in the SEV signaling pathway both upstream and downstream of Raf (Allard et al., 1996). Taken together, these results suggest also that during Drosophila development, RTK signaling cascades significantly deviate from a strictly linear pathway. This raises the question of which other components participate in signaling from the different receptors. Since *drk* was identified in a screen for modifiers of the rough-eye phenotype caused by a constitutively activated SEV receptor (Olivier et al., 1993), we set out to identify mutations in genes that define alternative routes of SEV signaling that act independently of direct DRK binding to SEV.

In this study, we describe the identification of a novel gene, *daughter of sevenless* (*dos*). We have identified mutations in *dos* as suppressors of activated SEV. *dos*

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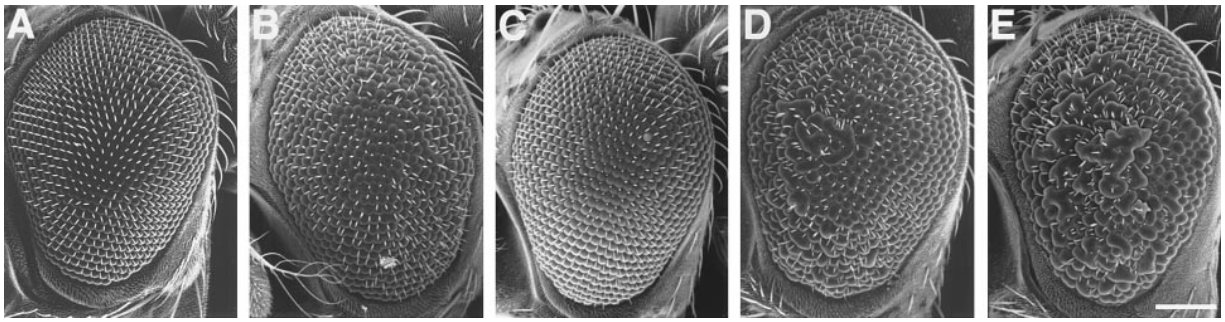


Figure 1. Interaction of Su(sev^{S11}) Mutations with sev^{S11} and $Ras1^{V12}$

Scanning electron micrographs of adult eyes of the following genotypes: wild type (A); sev^{S11} (B); sev^{S11} / $Su(sev^{S11})3A$ (dos^{R31}) (C); $Ras1^{V12}$ (D); $Ras1^{V12}/Su(sev^{S11})3A$ (E). Suppression of the rough-eye phenotype of sev^{S11} flies (B) by dos^{R31} (C). dos^{R31} and dos^{P115} mutations interact with sev^{S11} but do not suppress the rough-eye phenotype of Ras^{V12} (D; E), placing *dos* function upstream or independent of *Ras1*. The scale bar indicates 100 μm.

encodes a protein with all the characteristic features of a multiadaptor protein. It is required for signal transduction from SEV and other RTKs in Drosophila. We show that DOS acts upstream or independently of Ras1 and that its association with the membrane is dependent on the activity of the SEV receptor. DOS appears to be an essential component in the multimeric complex that is formed around the activated receptor.

Results

A Genetic Screen to Identify Signaling Components Downstream of the Sevenless RTK

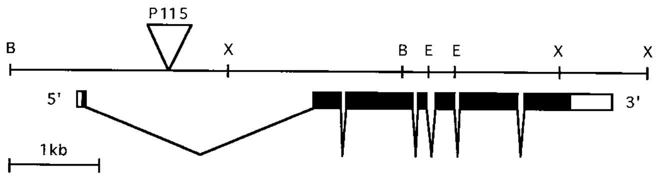
The eye of Drosophila is composed of approximately 800 ommatidia, each containing eight photoreceptor cells (R1–R8), four lens-secreting cone cells, and a number of pigment cells (reviewed by Wolff and Ready, 1993). The specification of one of the eight photoreceptor cells, R7, is dependent on activation of the SEV RTK by the Bride of Sevenless (BOSS) protein expressed in the neighboring R8 cell (Krämer et al., 1991). Cone and R7 precursor cells are referred to as the R7 equivalence group (Greenwald and Rubin, 1992), since in the absence of SEV activity, the R7 precursor cells fail to initiate neural development and develop as nonneuronal cone cells (Tomlinson and Ready, 1986). Conversely, cone cell precursors can be triggered to become R7 cells by constitutive activation of SEV. Constitutive activation of SEV has been achieved either by removing the extracellular domain of SEV (sev^{S11}; Basler et al., 1991) or by fusing the cytoplasmic domain of SEV to the transmembrane and extracellular domains of a dominant gain-of-function form of the Torso RTK (Tor^{4021}–*sev*; Dickson et al, 1992b; Sprenger and Nüsslein–Volhard, 1992). It is possible to drive the expression of these transgenes in the eye imaginal disc with one or two copies of the *sev* enhancer (*sE*) and therefore mimic the expression pattern of the endogenous SEV protein. Transgenic animals carrying these constructs have rough eyes owing to the recruitment of additional R7 cells (Figures 1A and 1B). This phenotype can be modulated by increasing or decreasing the activity of downstream components in the signal transduction pathway. In this way, mutations in genes for such components can be identified in the heterozygous state, thus circumventing the problem of identifying signaling components that are shared by different RTKs and, consequently, proving essential for viability of the fly.

More than 100,000 progeny of a cross between mutagen-treated males and females homozygous for the sev^{S11} transgene were individually screened for modification of the rough-eye phenotype (Figure 1C). In a second screen, more than 3,000 lethal P-element insertions on either the second or third chromosome (Török et al., 1993; P. D., unpublished data) were crossed to sev^{S11} flies, and the progeny was scored for modification of the sev^{S11} phenotype. Suppressors ($Su[sev^{S11}]$) that show the most consistent phenotype were further characterized by complementation analysis and fine-scale mapping. In addition to alleles of genes whose products are known to act downstream of SEV, such as DRK, SOS, and Ras1, we have identified two alleles (*R31* and *P115*) of a novel lethal complementation group $Su(sev^{S11})3A$. Owing to the proposed function of $Su(sev^{S11})3A$ in SEV signal transduction (see below), we named this mutation *daughter of sevenless*.

DOS Encodes a Potential Multiadaptor Protein with a Pleckstrin Homology Domain

dos^{P115} contains a single P-element insertion at position 62F on the left arm of the third chromosome. Mobilization of the P-element resulted in reversion of the lethality associated with this insertion and the suppression of sev^{S11} (see Experimental Procedures). To clone the *dos* gene, we recovered approximately 4.8 kb of genomic DNA flanking the dos^{P115} insertion site. Northern blot analysis with RNA extracted from different developmental stages with the genomic fragments revealed a single transcript of 3.2 kb that was preferentially expressed during early stages of embryonic development and in late larval or early pupal stages (data not shown). Embryonic and imaginal disc cDNA libraries were screened with the genomic probe, and several cDNAs were isolated. Restriction mapping and Southern blot analysis confirmed that all cDNAs belonged to the same transcription unit. The genomic organization of the *dos* gene shown in Figure 2A was determined by comparing the sequence of cDNA and genomic clones. We mapped

A



B

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  1 MDRTFYEGWL IKSPPTKRIW RARWRRRYFT LKQGEIPEQF CLEYYTDHNC
 51 RKLKGVIDLD QCEQVDCGLR LENRKQKFQY MFDIKTPKRT YYLAAETEAD
101 MRDWVNCICQ VCHLHDTKQS NELPLGAVGA DENRTQHTSS SGGLSNSTQN
151 TTTTSLHSSA GTTAPQASVP NAGGSAQLRR PAVIEEQPMP SNAGNNNSDS
201 VYVNTEYSNR ETMLCDANFD QQELLSAAQQ QPPPSPATAL YLNHSALIQA
251 QAAAAAAEQL QQQQQAARL AVSANGVVRK LPEHLVLTQQ TLAEAAAQQH
301 SSVQASPALS TASGPYIPIS ECFSGSPRFL PGVPLPGADL AMPNNPTTPL
351 NNLDPKFYDT PRSHNNIGLN LTNDQSYSPK ITNLSLQQLA NNNASKQRSD
401 SDSESVFTDD DEWAHPLPLR ENVDRSTRPS DSSIENESFV LTYSQRFSKM
451 PEEGGAIVPP AEKSSKLAGA ASLAEAGDQG TLDKLAKVLK NKNNLILDFK
501 ENEKIPRDLP QLSDTENTSP AIVARRNAHS AFIEESYDIP RSHQQPYYNV
551 NQLLGERPVT SPHNSNPIAA STPNLMAADL GAVAAISAAA NPGLMGEAQA
601 VASSPTSART LPRHCYTNAA PTKMEGNVFR YDFMEQADCP PVNRKLKPKV
651 AGGLPVVEDK PPEEFPAKPP VGVDQLTNKL GAAQLQQPIG PPSVDRKCKP
701 NAYKLGNSAT MSPATRRSSG APLSMVLPHE TDVHSPAAAN AFFHETRTLP
751 RQQHRHHPNS PGSMSVQHQR TASAAAAMMS LTAAAAPKQQ AAAQTEHKLQ
801 YFDLDVTNKP PLLNRSSMSV GNLYSQGGNG ASGMRFAGVE AGGARAPVPS
851 SVVYRSVDFV KTEAFKRIRE ERESSGNK
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C

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           I               II                  III             IV
        **** *         ** *** *             * ** *       ******  *  * *
Dos   6 YEGWLIKSPPTKRIWRARWRRRYFTLKQGEIPEQF.CLEYYTDHNCRKLKGVIDLDQCEQVDCGL
        : ||| |||| |:: |  |:||:| |: | : ::  |||| : : :|  :|||: |:||| ||
Gabl  8 CSGWLRKSPPEKKLKRYAWKRRWFVLRSGRLTGDPDVLEYYKNDHAKKPIRIIDLNLCQQVDAGL

                   V                VI
                  * * *       * **** **  **  *   *
Dos  70 RLENRKQKFQYMFDIKTPKRTYYLAAETEADMRDWVNCICQVCHLHDT 117
         :: :     |:||| |   | :|| |: | :|  || |||::| ::.|
Gabl 73 TFNKKEFENSYIFDINTIDRIFYLVADSEEEMNKWVRCICDICGFNPT 120
```

Figure 2. Molecular Characterization of the *dos* Gene

(A) Map of the *dos* genomic structure. The horizontal line represents the genomic DNA. Restriction sites for BamHI (B), EcoRI (E), and XhoI (X) are indicated. The insertion site of the P element in dos^{P115} maps to the first intron. Below, the *dos* cDNA is diagrammed with closed boxes representing coding regions, whereas open boxes represent untranslated sequences.

(B) Predicted amino acid sequence of the DOS protein. The PH domain is represented by broken lines. Tyrosine residues and flanking sequences that match the consensus binding sites for SH2 domain–containing proteins are underlined (Songyang et al., 1993, 1994). For details, see Discussion. The RxxPxxP motif shown in bold characters (position 328–334) is indicative for SH3-domain binding (Pawson, 1995).

(C) Comparison of the DOS and Gab1 PH domains (Holgado-Madruga et al., 1996). Identical and similar amino acids are indicated by vertical bars and colons, respectively. I-VI represent the six conserved subdomains of the PH domain (Musacchio et al., 1993). Amino acids of DOS that conform the consensus sequence within the subdomains are labeled with asterisks.

the insertion site of the P element in dos^{P115} to the first intron. Since the translation start site of *dos* lies in the first exon (Figure 2A), dos^{P115} is likely to be a complete loss-of-function allele.

To confirm that the mutant phenotypes we observed were due to mutations in the isolated gene, we placed the complete cDNA under the control of the *sev* enhancer and *hsp70* promoter sequences (*sE–dos*) and generated transgenic flies. Repeated heat-shock induction of the transgene during development was sufficient to rescue the lethality of homozygous dos^{R31} and dos^{P115} or transheterozygous dos^{R31}/dos^{P115} animals. The surviving flies were fully viable and fertile. Since mutations in *dos* were isolated as dominant suppressors of the multiple-R7 phenotype caused by the sev^{S11} construct (Figures 3B and 3C), expression of the *dos* transgene under the *sev* enhancer should revert this phenotype. Indeed, one copy of the *sE–dos* construct was sufficient to restore the multiple-R7 phenotype in $sev^{S11}/+;dos^{R31}/$ *sE-dos* flies (Figure 3D).

The size of the longest cDNA (3184 bp) corresponds to the 3.2 kb transcript detected on Northern blots. It contains a single open reading frame of 2634 bp with in-frame stop codons preceding the first potential initiation codon at position 46. This cDNA encodes a novel protein of 878 amino acids with a predicted molecular mass of 96 kDa (see Figure 2B). Analysis of the protein sequence (see Figure 2B) identified an amino-terminal pleckstrin homology (PH) domain (Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993), a polyproline motif

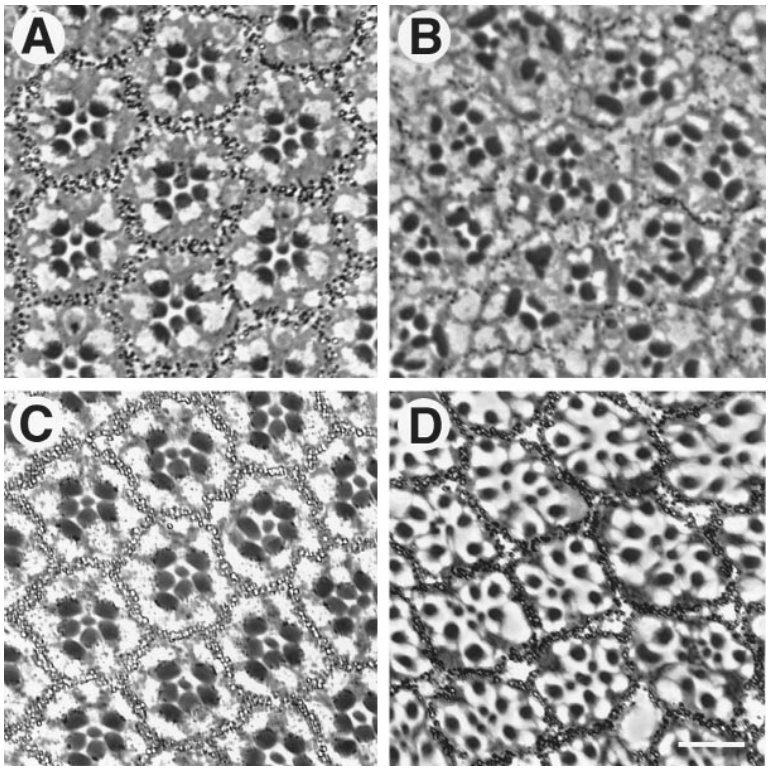

Figure 3. Expression of *dos* under Control of the *sev* Enhancer Reverts the Suppression of the sev^{S11} Rough-Eye Phenotype by dos^{R31}

Histological sections of the eyes of wild type (A), sev^{S11} (B), sev^{S11}; dos^{R31}/+ (C), and sev^{S11}; dos^{R31}/*sE–dos* (D) flies are shown. The recruitment of multiple R7 cells in ommatidia of sev^{S11} flies (B) is suppressed by the dos^{R31} mutation (C). This suppression is reverted by expression of the *dos* cDNA under the control of the *sev* enhancer sequences (D), indicating that the cDNA encodes a functional DOS protein. The scale bar indicates 10 μm.

RxxPxxP (amino acids 328–334) indicative for SH3 domain binding, and, most strikingly, 10 potential tyrosine phosphorylation sites with consensus sequences for binding SH2 domains (Songyang et al., 1993; 1994). These functional motifs are similar to those described for the mammalian insulin receptor substrates IRS–1 and IRS–2 and the EGF and insulin receptor substrate Gab1 (Holgado–Madruga et al., 1996; Sun et al., 1993, 1995). Although the sequence homology between DOS and these proteins is low outside the PH domain (see Figures 2B and 2C), the conservation of structural motifs suggests that DOS, Gab1, IRS–1, and IRS–2 form a family of multiadaptor proteins that link RTKs to downstream signaling molecules containing SH2 and SH3 domains. To characterize the DOS protein, we generated polyclonal and monoclonal antibodies against a bacterially synthetized GST–DOS fusion protein. The antibodies recognize a prominent band of 115 kDa on a Western blot of protein extracts from wild-type larvae. This band was strongly induced in extracts from larvae carrying an *sE–dos* transgene when these larvae were heat-shocked (data not shown).

DOS Acts Downstream of SEV but Upstream of Ras1

The primary structure of the DOS protein suggests that it acts as an adaptor at the cell membrane for various proteins with SH2 domains. It may therefore serve to couple SEV to different signaling pathways. In order to characterize the function of DOS, we wanted to answer the following questions. First, does DOS indeed act between SEV and Ras1 or Raf, as suggested by its structure? Second, if so, where does it act with respect to the function of DRK and Ras1? Third, where within the cell is DOS localized? Is it associated with the membrane, as suggested by the presence of a PH domain? Fourth, is the subcellular distribution of DOS altered upon RTK activation? Fifth, is DOS function only required downstream of SEV, or is it also required for signal transduction via other RTKs during development? In the following, we will address each of these questions separately.

To test where in the SEV pathway DOS acts, we performed genetic epistasis experiments. If DOS indeed functions downstream of SEV and upstream of Ras1, as expected from its sequence, *dos* mutations should suppress activating mutations in *sev* but not in *ras1* or *raf*. Transgenes that encode constitutively active versions of Ras1 ($Ras1^{V12}$; Fortini et al., 1992) and Raf ($raf^{tor4021}$; Dickson et al., 1992a), expressed under the control of *sev* regulatory sequences, exhibit similar phenotypes to those of the constitutively activated SEV. Although mutations in *dos* strongly suppress the rough-eye phenotype caused by the activation of SEV, they fail to suppress the phenotype caused by activation of Ras1 or Raf (compare Figures 1B and 1C with Figures 1D and 1E). This result provides genetic evidence for a function of DOS between SEV and Ras1.

The products of four genes, DRK, SOS, CSW, and now DOS, have been shown to be required for signaling downstream of SEV and upstream or in parallel to Ras1 (Simon et al., 1991, 1993; Olivier et al., 1993, Allard et al., 1996). To find out whether these proteins act together or in separate signaling pathways, we tested their requirement for signaling from a mutant SEV receptor that is unable to bind DRK. We have previously identified the tyrosine residue Y2546 on the SEV receptor that is

Table 1. Interaction of *dos*, *drk*, *Ras1* and *csw* Mutations with an Activated SEV RTK That Lacks the Direct Binding Site for the Adaptor Protein DRK ($torso^{4021}$–sev^{Y2546F})

		Percentage of Ommatidia with Extra R7 Cells
$torso^{4021}$–sev^{Y2546F}/Y	+/+	93 ± 2.0
$torso^{4021}$–sev^{Y2546F}/Y	dos^{R31}/+	67 ± 8.9
$torso^{4021}$–sev^{Y2546F}/Y	drk^{R1}/+	38 ± 5.7
$torso^{4021}$–sev^{Y2546F}/Y	$Ras1^{e2F}$/+	58 ± 5.3
$torso^{4021}$–sev^{Y2546F}/+	+/+	43 ± 4.5
$torso^{4021}$–sev^{Y2546F}/+	$csw^{E(sev)1A}$	5 ± 0.6

Due to the cold-sensitive phenotype of the $torso^{4021}$ mutation (Szabad et al., 1989), the experiment was carried out at 18°C to enhance the multi-R7 cell phenotype. At 25°C, $torso^{4021}$–sev^{Y2546F} flies possess smooth eyes with a low number of R7 cells (Raabe et al., 1995). The values are given as the percentage of ommatidia with more than one R7 cell. For each cross, between five to eight eyes were sectioned, and between 100 and 200 ommatidia were analyzed in each eye. The experiments were performed in a w^{1118}; sev^{d2} background.

critical for DRK binding to the receptor (Raabe et al., 1995). We have shown that a single amino acid substitution at this site abolishes detectable binding of DRK to an activated receptor (Torso^{4021}–sev^{Y2546F}). However, this mutant receptor is still able to induce R7 cell development, albeit at a reduced frequency (Raabe et al., 1995). To investigate whether DOS, DRK, or Ras1 are still required for signaling from this receptor, we introduced mutated versions of the corresponding genes into the $torso^{4021}$–sev^{Y2546F} background (Table 1). In this experiment, 93% of the ommatidia of $torso^{4021}$–sev^{Y2546F} flies contain more than one R7 cell in the presence of two wild-type copies of these genes. Removal of one copy of the *Ras1* gene resulted in a significant reduction in ommatidia with multiple R7 cells. Therefore, *Ras1* function is still critical for signaling from this mutant receptor. Similarly, the multiple-R7 phenotype is also suppressed by removing one copy of *dos*. Like Ras1, DOS plays a rate-limiting role in signaling by the Torso^{4021}–sev^{Y2546F} receptor in the absence of direct binding of DRK to the receptor. Surprisingly, we found that the *drk* mutation also significantly suppressed the multi-R7 phenotype. The reduction in the amount of functional DRK protein reduces the efficiency of signaling by Torso^{4021}–sev^{Y2546F} receptor even though direct binding of DRK to this receptor is prevented. This suggests that DRK has multiple functions in SEV signaling. In addition to direct binding to SEV, DRK may also function by binding to other proteins. It is interesting to note that DOS has several putative DRK SH2 domain binding sites. A dominant negative form of the Corkscrew (CSW) tyrosine phosphatase (Perkins et al., 1992) that has been isolated as an enhancer of a hypomorphic *sev* allele (Simon et al., 1991; Allard et al., 1996) exhibits a very strong interaction with $torso^{4021}$–sev^{Y2546F} (Table 1). We conclude that DOS, DRK, Ras1, and CSW are required for signaling from the mutant SEV receptor lacking a DRK binding site.

Localization of DOS in the Developing Drosophila Eye

The genetic and molecular data presented above suggest a role for DOS close to the SEV receptor. To determine the expression pattern in the developing eye and

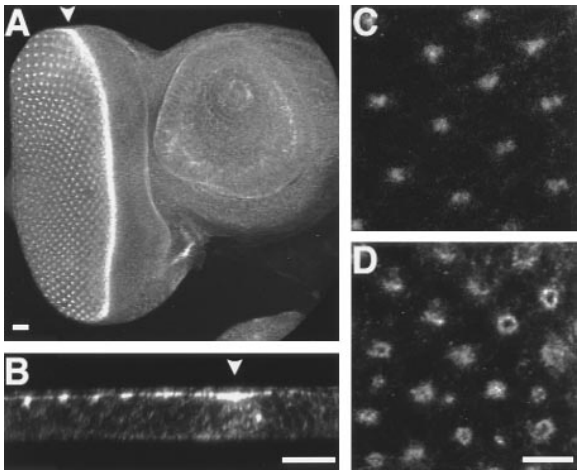

Figure 4. Expression Pattern of DOS in the Eye Imaginal Disc

(A–D) Confocal images from eye-antennal imaginal discs stained with a monoclonal antibody against the DOS protein.

(A) The DOS protein is preferentially expressed in the morphogenetic furrow (arrowhead) and during assembly of the ommatidial clusters posterior to the furrow. A general weak staining is observed in the remaining disc with higher levels of staining in cells that give rise to the ocelli.

(B) The same eye imaginal disc in an apical–to–basal cross section. The DOS protein is localized apically both in the morphogenetic furrow (arrowhead) and in the developing ommatidial clusters posterior to the furrow.

(C) Higher magnification of (A) at a level 12–16 rows behind the morphogenetic furrow. Membrane localization of DOS is restricted to the developing photoreceptor cells in the most apical part of the cell. In comparison, an eye imaginal disc from an sev^{S11} larva (D) in which the staining and localization pattern of DOS essentially overlaps with the expression pattern of the SEV RTK as described in Tomlinson et al. (1987). Anterior is to the right. The scale bar in (A) indicates 200 μm, in (B) and (D) 20 μm.

the potential site of action of DOS within the cell, we stained eye imaginal discs with the monoclonal antibody to DOS. Although low levels of staining were observed in all cells of the eye imaginal disc, a significant accumulation of DOS was detected at the membranes of cells in the morphogenetic furrow and in clusters of cells behind the furrow (Figure 4A). Owing to the apical constriction of the photoreceptor cells, it was not possible to identify unambiguously the cells that contained high levels of membrane-associated DOS. Membrane-localized DOS was restricted to a small region just below the apical surface of these cells (Figure 4B). It is likely that this region corresponds to the adherens junctions, since staining with an antibody to Armadillo, which specifically stains these junctions, labeled the same region of the cell membrane (data not shown). The subcellular localization of DOS is similar to that of the DRK adaptor protein (Olivier et al., 1993), though DRK is membrane-associated in all cells of the eye imaginal disc. DOS protein, however, was only located at the membranes of cells within and immediately posterior to the morphogenetic furrow, where ommatidial cluster formation was initiated. Further behind the furrow, where the more mature clusters were located, DOS was only membrane-associated in a few cells in each ommatidial cluster. Interestingly, the localization of DOS was altered in sev^{S11}

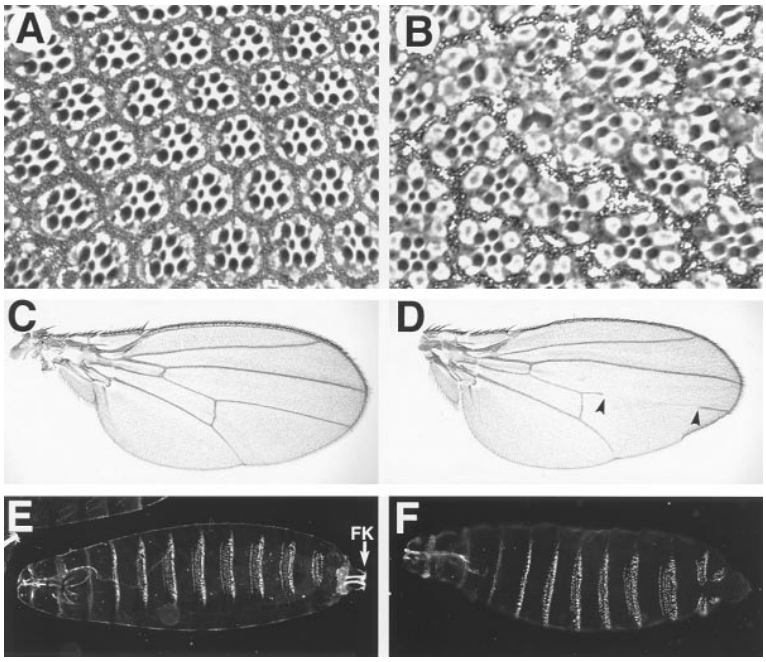

Figure 5. DOS Is Required for Normal Development in the Embryo, Wing, and Eye

(A, B) Histological sections through a wild-type eye (A) and an eye carrying a homozygous dos^{R31} clone (B) generated by FLP induced mitotic recombination in a heterozygous mutant background. The clone is marked by the absence of pigment granules normally present in pigment and photoreceptor cells. Photoreceptor cells lacking pigment granules were occasionally observed but were greatly underrepresented.

(C, D) Phenotype of a dos^{R31} mutant clone in the wing generated by FLP induced mitotic recombination. A wild-type wing is shown in (C). The clone extends between the two arrowheads in (D), removing part of vein L4.

(E, F) Dark-field photographs of cuticular preparations of wild-type embryos (E) and embryos derived from dos^{R31} germline clones (F). The embryos in (F) received a wild-type copy of *dos* from the father but lacked maternally derived dos function. (F) shows the most severe phenotypes observed with a reduced head skeleton and the most posterior structures, including the Filzkörper and abdominal segments A7 and A8 missing or malformed (FK: Filzkörper).

eye imaginal discs, where the SEV RTK was constitutively activated in all *sev*-expressing cells, including the R7 precursor cells, mystery cell, and the cone cell precursors (compare Figures 4C and 4D). In these discs, the localization of DOS resembled that of the SEV protein. SEV accumulation in the cone cell precursors results in ring-like staining patterns in clusters located 12–14 rows behind the morphogenetic furrow (Tomlinson et al., 1987). Since we observed DOS staining in similar ring-like patterns at the same position in sev^{S11} discs, we assume that the altered localization of DOS is caused by activation of the SEV RTK in the cone cell precursors. This suggests that DOS becomes membrane-associated in the eye imaginal discs in response to SEV RTK activation.

DOS Is Required for Signaling by Various RTKs

In addition to its role in the SEV pathway, DOS appears to be required at earlier stages of development, since complete loss of *dos* function causes lethality at the third larval instar stage. Imaginal discs from these animals were severely reduced in size or completely missing (data not shown). This phenotype is similar to that of homozygous mutants of other signaling molecules that act downstream of RTKs and suggests that DOS is also required for the proliferation or survival of cells in imaginal discs. Since many signal transduction components have been shown to be shared by different RTKs, we examined the effect of *dos* mutations in well-defined assays for signaling by the EGF receptor homolog (DER) in the eye and wing and by Torso in the embryo.

We generated clones of cells homozygous for dos^{R31} or dos^{P115} by mitotic recombination. In the eye, cells homozygous for dos^{R31} or dos^{P115} rarely differentiated as photoreceptor cells, indicating that DOS is required for the development of all photoreceptor cells (Figures 5A and 5B). Similar results have been obtained for mutations in other genes of the Ras/Raf signaling cassette (Simon et al., 1991, Dickson et al., 1992a). In the wing, cells homozygous for *dos* mutations were smaller in size and unable to differentiate vein structures (Figures 5C and 5D). This is consistent with a role for DOS as a mediator of DER signaling in the wing, since loss-of-function mutations in DER or its identified downstream signaling components show similar phenotypes (Diaz–Benjumea and Hafen, 1994). Further evidence that the DOS protein is involved in DER signaling in the eye and the wing has been obtained by genetic interaction experiments with a dominant gain-of-function allele of *DER*, *DERElp* (Baker and Rubin, 1989). The eye and wing phenotype of these flies was partially suppressed by *dos* mutations (data not shown).

To analyze the effect of *dos* mutations on Torso signaling in the early embryo, we used the "dominant female-sterile" technique in combination with the hsFlp/Flp-recombinase-target system (FRT) (Chou et al., 1993; Hou et al., 1995), which allows the efficient recovery of germline clones. Embryos derived from female germ cells that were homozygous for either of the two *dos* alleles but contained a wild-type copy of *dos* from the father exhibited very distinct defects in their terminal structures. At the posterior, these animals rarely differentiated Filzkörper, and the abdominal segment A8 was frequently affected; at the anterior, the head skeleton was reduced in size in the majority of animals (Figures 5E and 5F). Lack of terminal structures was also observed in the absence of Torso function. Strikingly, however, the observed phenotypes were consistently weaker than the phenotype of loss-of-function mutations in *torso* or *raf*. In these mutants, the terminal structures, including A8, were always missing. However, embryos that lack DRK, SOS, and Ras1 activity also exhibit weaker phenotypes than those of *torso* and *raf* loss-of-function mutants (Hou et al., 1995). Since dos^{P115} is likely to be a

complete loss-of-function allele, we assume that, as in the case of *drk*, *Sos*, or *Ras1* embryos (Hou et al. 1995), there is residual activity in the Torso pathway in the absence of *dos* function. Embryos lacking both maternal and zygotic function of *dos* possessed hardly any cuticular structures, suggesting that *dos* function was also required at later stages of embryogenesis. In summary, we have shown that DOS acts positively in the signaling process driven by a number of distinct RTKs that function at different stages in Drosophila development.

Discussion

In the search for components of the SEV RTK signaling pathway, we have identified mutations in a novel gene, *dos*, that encodes a novel protein with sequences characteristic of multisite docking proteins. Like most other known components of the Ras1 signaling pathway, DOS is required repeatedly during development, and loss of *dos* function is lethal. Analysis of *dos* mutant clones in the germline and the wing and of genetic interactions with *sev* and *DER* mutants indicates that DOS is an essential component for signaling from the Torso, DER, and SEV receptors.

DOS May Act as a Multiadaptor Protein

Several lines of evidence suggest that DOS functions as a multiadaptor protein in RTK signal transduction. The results of our genetic analysis in the SEV pathway indicate that DOS acts downstream of the receptor but upstream or in parallel to Ras1 and Raf. Reduction in the gene dosage of *dos* suppresses the rough-eye phenotype caused by activated SEV receptor but not that caused by activated Ras1 or activated Raf. The amino acid sequence of DOS contains multiple consensus SH2 domain binding sites. Thus, in response to receptor stimulation, DOS may become tyrosine-phosphorylated at multiple sites and act as a docking protein for SH2 domains. Residues carboxy-terminal to the phosphotyrosine are critical to create specific binding motifs for different SH2 domains (Songyang et al., 1993, 1994). Analysis of the amino acid sequence of DOS reveals consensus sequences for the SH2 domains of DRK/Grb2 (YXNX at positions 202, 207, 241, 547, and 616), PLC-γ (YDTP position 358, YDIP position 537), the regulatory subunit of Phosphatidylinositol-3-kinase (YXXM position 631) and Shc (YIPI, position 316). Additionally, the YFDL motif at position 801 is very similar to YIDL, the binding site for the protein tyrosine phosphatase Syp on the IRS–1 protein (Sun et al., 1993). Syp is the mammalian homolog of Drosophila *CSW*, which has been implicated in Torso and SEV mediated signaling (Perkins et al., 1992; Feng et al., 1993; Vogel et al., 1993; Allard et al., 1996). Indeed, Herbst et al. (1996 [this issue of *Cell*]) have identified DOS as a tyrosine-phosphorylated protein that is tightly bound to a dominant negative form of CSW in Schneider cells (a Drosophila embryonic cell line). They have further demonstrated that the interaction between DOS and CSW is mediated by the SH2 domains of CSW. The corresponding mutant allele of *csw* acts as a strong suppressor of the rough-eye phenotype of sev^{S11} flies.

The proposed role for DOS is analogous to the function of the docking proteins IRS–1, IRS–2, and Gab1 in vertebrates (Sun et al., 1993, 1995; Skolnik et al., 1993; Holgado–Madruga et al., 1996). In addition to the presence of multiple SH2 binding consensus sequences, DOS shares two other regulatory motifs with these proteins: a polyproline region and a PH domain. A type I class of polyproline motif (RxxPxxP residues 328–334) indicates that DOS has the potential to interact with SH3 domain–containing proteins (Pawson, 1995). PH domains have been identified in a large number of signaling proteins (Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993) and have been implicated in membrane association either by interacting with membrane-bound proteins or by binding directly to phospholipids (reviewed by Shaw, 1996). The localization of the DOS protein at the membrane of developing photoreceptor cells is consistent with this view and may be a prerequisite for DOS to act as a substrate for membrane-bound receptors. In eye discs of sev^{S11} larvae, SEV RTK is constitutively activated in all cells in which the endogenous SEV protein is expressed (Tomlinson et al., 1987), and DOS membrane localization closely resembles the distribution and localization of SEV. This indicates that DOS localization to the membrane may be controlled by RTK activation. Its dynamic association with the plasma membrane and the multiple protein docking sites suggests that DOS serves as a matrix for the assembly of different signaling components around the receptor.

Growing Complexity of RTK Mediated Signaling

Biochemical and recent genetic evidence suggests that activated RTKs activate multiple effector pathways (Hou et al., 1995; Karlovich et al., 1995; McCollam et al., 1995; Raabe et al., 1995). In the Torso pathway, the effects of the complete absence of different signaling components can be compared directly, since this is the first time they are required during embryogenesis. Loss-of-function mutations in *dos*, *drk*, *Sos*, and even *Ras1* produce weaker phenotypes than the loss-of-function mutations of *torso* or *raf*, respectively (Hou et al., 1995; this study). This indicates that Torso can signal to Raf by a yet unknown pathway. Whether DOS is part of this parallel pathway or whether it participates only in the Ras1-dependent pathway is unclear. This question could be addressed by simultaneous removal of DRK and DOS activities in the germline.

In the SEV pathway, the effects of complete removal of individual signaling components cannot be analyzed, owing to their previous requirement in cell proliferation. Nevertheless, altering the gene dosage of individual signaling components in flies carrying activated SEV receptors indicates that the activation of Ras1 does not follow the simple linear pathway outlined in the Introduction. Although the elimination of the unique DRK binding site on the SEV receptor impairs signaling, it does not eliminate it (Raabe et al., 1995). Interestingly, we find that signaling from this mutant receptor is still dependent on wild-type levels of DOS, DRK, and Ras1. This suggests that DRK functions as an adaptor not only to couple SOS to the receptor but also as an adaptor between DOS and SOS. In addition, DOS also serves as

a docking site for the tyrosine phosphatase CSW (Herbst et al., 1996). A dominant negative mutant allele of *csw* strongly suppresses the rough-eye phenotype of *tor*^{4021}–*sev*^{Y2546F}. This is consistent with the view that CSW participates in the signaling process by interacting with DOS directly. Since CSW exhibits a stimulating rather than an inhibiting role in SEV signaling (Allard et al., 1996), it is expected that the dephosphorylation of some sites on DOS may be required for altering its adaptor function (Herbst et al., 1996). The elimination of individual protein binding sites on DOS by mutations will be required to identify the role of these various adaptor sites in the SEV and the Torso pathway. Such an approach may permit the functional dissection of this increasingly complex signaling cascade.

Experimental Procedures

Genetics

Fly cultures and crosses were performed according to standard procedures. Suppressors of the rough-eye phenotype of *sev*^{S11} (Basler et al., 1991) were isolated as follows. Isogenized males of the genotype *w*^{1118}, *sev*^{d2} were fed 25mM ethyl methanesulfonate as described (Lewis and Bacher, 1968) and crossed with virgin females homozygous for the *sev*^{S11} transgene on the third chromosome. Approximately 100,000 progeny flies were screened for suppressors of the rough-eye phenotype, and initially more than 50 suppressor lines *Su(sev*^{S11}*)* were established. Only lines that gave reproducible results were pursued. The mutations were mapped meiotically by crossing to flies carrying multiple marked chromosomes (*b, pr, cn, bw* for the second chromosome and *th, st, cu, sr, e, ca* for the third chromosome). Virgin females carrying the mutant chromosome over the multiple marked chromosome were mated with *sev*^{S11} males. The progeny was scored for suppression, and those flies were individually backcrossed to the multiple marked strain to look for the presence of individual markers on the recombinant chromosomes. Further characterization was done by complementation analysis with deficiencies and the *E(sev)* loci (Simon et al., 1991). The same screening procedure was also applied to collections of lethal P-element insertion lines on the second (Török et al., 1993) and third chromosomes (P. D., unpublished data). The P-element insertion sites were localized by in situ hybridization to polytene chromosomes. P-element excision lines were generated using a stable source of transposase (Robertson et al., 1988) to confirm that the P-insertion was responsible for suppression and associated lethality.

The following fly strains were used to test for genetic interactions of the isolated suppressors: *Elp*^{B1} (Baker and Rubin, 1989) and *RasV12* (Fortini et al., 1992).

To measure the effect of *dos, drk, Ras1*, and *csw* mutations on the ability of a mutated SEV receptor (*sE-torso*^{4021}–*sev*^{Y2546F}; Raabe et al., 1995) to specify R7 cells, males carrying these mutations over a balancer chromosome were crossed to females of the genotype *w*^{1118}, *sev*^{d2}, *sE-torso*^{4021}–*sev*^{Y2546F}. The number of R7 cells per ommatidium was scored in the male progeny with the exception of *csw*, in which females were analyzed. At least five eyes were sectioned for each cross. The eye phenotype of flies carrying the *sE-torso*^{4021}–*sev*^{Y2546F} construct is temperature-dependent. At 25°C, flies carrying the construct in heterozygous condition have smooth eyes (Raabe et al., 1995). At 18°C, the temperature chosen in this study, flies of the same genotype had rough eyes with multiple R7 cells.

To test whether ubiquitous expression of the *sE–dos* transgene can rescue the lethality of *dos* mutations, *sE–dos/+; dos*^{R31}/*dos*^{P115} animals were shifted to 37°C for 60 min every 6 hr during development. Rescue was also observed for homozygous *dos*^{R31} and *dos*^{P115} animals.

Clonal Analysis

Germline clones of *dos* alleles were generated using the autosomal dominant female–sterile technique in combination with the Flp recombinase system (Chou et al., 1993; Hou et al., 1995). Females of the genotype *w*^{1118}*; dos*^{R31}*–FRT80B* were crossed with *hsFlp1/Y; P(ovo*^{D1}*)–FRT80B* males. First instar larvae were heat-shocked for 1 hr at 37°C. Females of the genotype *hsFlp1/+; P(ovo*^{D1}*)-FRT80B/ dos*^{R31}*–FRT80B* were selected and crossed either with wild-type males to provide zygotic dos function or with *dos*^{R31}*/TM6B* males to select for embryos that lacked any dos activity. The phenotypes of embryos derived from these females were examined 5 days after egg laying. Somatic clones in the eye and in the wing were induced in first instar larvae of the genotype *hsFlp1; dos*^{R31}*–FRT80B/ P(w*$^{+}$*)75C, FRT80B* as described by Xu and Rubin (1993).

Molecular Analysis

Standard recombinant DNA procedures were done as described in Sambrook et al. (1989). Genomic sequences flanking the *dos*^{P115} insertion site were cloned by plasmid rescue (Mlodzik et al., 1990). The 3.0 kb EcoRI rescue fragment was used to screen a Drosophila eye-antennal disc cDNA library (A. Cowman, unpublished data) and an embryonic (4–8 hr) cDNA library provided by M. Noll (Zürich, Switzerland). Genomic clones encompassing the *dos* gene locus were recovered from a λGEM11 library (K. Kaiser). The sequence of cDNA and genomic subclones was obtained after generation of a series of deletions using the nested deletion kit (Pharmacia). Sequencing was done on dsDNA templates using the DyeDeoxy™ Terminator Cycle Sequencing kit (Perkin Elmer). Sequence data were assembled and analyzed using the Staden and Genetics Computer Group software packages. All cDNA clones were sequenced on both strands.

Plasmid Construction and Germline Transformation

The *sE–dos* rescue construct was produced by cloning the complete *dos* cDNA as an Asp718–NotI fragment into a modified pW8 vector (Klemenz et al., 1987).

The *sE-torso*^{4021}–*sev*^{Y2546F} construct is described in Raabe et al. (1995). All constructs are under the control of the *hsp70* promoter and a single or duplicated 1.2 kb *sev* enhancer element (Basler et al., 1991). Transgenic lines were generated by injecting Qiagen-purified plasmid DNA into *w*^{1118} or *w*^{1118},*sev*^{d2} embryos as described in Basler et al. (1991). Several independent transformant lines were established for each injected construct.

Generation of Antibodies and Immunohistochemistry

A cDNA fragment encoding amino acids 38–878 of DOS was cloned into a pGEX expression vector (Pharmacia). GST–DOS fusion protein expression in bacteria was induced with 1mM isopropyl-β-D-thiogalactopyranoside for 4 hr, and the purified fusion protein was used to generate monoclonal and polyclonal antisera in mice. Eye imaginal discs of wild-type or *sev*^{S11} larvae were fixed essentially as described in Gaul et al. (1992) and incubated overnight with a 1:10 dilution of anti-DOS supernatant or a 1:200 dilution of an anti-Armadillo serum (gift from A. Bejsovec). After washing in PBT (phosphate-buffered saline plus 0.3% Triton X-100), the discs were incubated for 1 hr with fluoroscein isothiocyanate– or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch), washed again, and embedded in phosphate-buffered saline/Glycerol. Confocal images were taken with a Multiprobe 2001 confocal laser scanning microscope (Molecular Dynamics). All figures were assembled in Photoshop (Adobe) and printed on a Pictrography 3000 (Fuji) digital printer.

Histology and Scanning Microscopy

Scanning electron microscopy and histological sections of eyes were done as described previously (Basler et al., 1991). To visualize the cuticle phenotypes, embryos were dechorionated, mounted in Hoyer's medium, and inspected under dark-field illumination.

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