Development 125, 5033-5042 (1998)
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DEV5244

small wing encodes a phospholipase C-γ that acts as a negative regulator of R7 development in *Drosophila*

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Accepted 2 October; published on WWW 12 November 1998

SUMMARY

Phospholipase C-γ (PLC-γ) is activated in many cell types following growth factor stimulation. Our understanding of the role of PLC-γ in cell growth and differentiation has been severely limited by the dearth of mutations in any organism. In this study, we show that the *Drosophila* gene *small wing* (*sl*), identified by Bridges in 1915, encodes a PLC-γ. Mutations of *sl* result in extra R7 photoreceptors in the compound eye, consistent with overactivation of the receptor tyrosine kinase pathways that control R7 development. The data presented here provide the first genetic evidence that PLC-γ is involved in Ras-mediated signaling and indicate that PLC-γ acts as a negative regulator in such pathways in *Drosophila*.

Key words: *Drosophila*, *small wing*, Phospholipase C-γ, Photoreceptor R7

INTRODUCTION

During cell growth and differentiation, growth factor receptors with tyrosine kinase activity trigger signal transduction cascades that ultimately lead to profound changes in cell behavior (reviewed by Kazlauskas, 1994). Phospholipase C-γ (PLC-γ) is an intracellular enzyme that is activated by many such receptor tyrosine kinases (RTKs), via an interaction between one of two SH2 (Src-homology 2) domains in PLC-γ with a specific phosphotyrosine on the intracellular part of the activated receptor (reviewed by Schlessinger and Ullrich, 1992). This association results in the phosphorylation of PLC-γ and an increase in its catalytic activity. PLC-γ is also activated indirectly by some receptors that do not themselves possess tyrosine kinase activity, such as the T cell antigen receptors and those for a wide variety of cytokines (reviewed by Rhee and Bae, 1997); in these cases PLC-γ may be activated by one of several non-receptor tyrosine kinases, including Src, Fyn, Lck, Lyn and Hck, that are activated by the receptor.

PLC-γ catalyzes the hydrolysis of phosphatidylinositol (4,5) bisphosphate into two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of Ca^{2+} from internal stores, thereby mediating a variety of cellular processes, including fertilization and cell growth (Berridge, 1993). DAG is an activator of protein kinase C (PKC), a serine/threonine kinase involved in a wide range of cellular activities, including responses to hormones, neurotransmitters and growth factors (reviewed by Nishizuka, 1995). Biochemical studies have presented a complicated picture as to the role played by PLC-γ in mitogenic signaling. In some cell types, there is evidence for a positive role, probably via activation of PKC, which is indicated by several studies to participate with Ras1 as an activator of Raf-1 (Burgering et al., 1993; Huang et al., 1995a; Cai et al., 1997). Other biochemical studies suggest a negative role, again via PKC (Seedorf et al., 1995; Obermeier et al., 1996). Adding a further twist to this complex picture are other studies, using microinjected PLC-γ, suggesting that an intact PLC-γ SH3 domain may be more important for mitogenic stimulation than the phospholipase activity of the protein (Smith et al., 1994; Huang et al., 1995b). Clearly, much has yet to be learned about what happens after PLC-γ activation in vitro as well as in vivo.

It is now clear that many RTK pathways do not pass their signal through a simple linear route (e.g. Kazlauskas, 1994; Schlessinger, 1994). Binding of the ligand to the receptor results in the phosphorylation of multiple tyrosines on the intracellular domain of many RTKs and each phosphotyrosine is recognized by one of several distinct SH2-containing proteins. In the case of the PDGF receptor for example, this includes adaptor proteins such as Nck, Shc and Grb2, enzymes such as phosphatidyl inositol 3-kinase (PI3K), PLC-γ, the GTPase activating protein of Ras (RasGAP), members of the Src family and the tyrosine phosphatase Syp (Kazlauskas, 1994). Simultaneous activation of several proteins by the same RTK suggests that multiple signals might be sent, which may explain some of the complexity in the role of PLC-γ in RTK-signaling (Kazlauskas, 1994). The emerging picture is one of a multiprotein complex being recruited to the activated receptor, from which multiple signaling

pathways radiate into the cytoplasm. Furthermore, crosstalk between the branches also occurs, with the effect that the cellular response to ligand binding is actually integrated from the competing effects of many proteins.

Genetic studies of RTK pathways that control cell fate determination in invertebrates have led to major advances in our understanding of RTK-mediated signaling (Dickson and Hafen, 1994). Three RTK pathways in *Drosophila* have received particular attention: Sevenless (Sev), which controls R7 photoreceptor cell development in the eye (reviewed by Zipursky and Rubin, 1994); the *Drosophila* EGF receptor homolog (DER), which is required during development of the oocyte and embryo, during wing vein differentiation and in all photoreceptor cells (R1-R8) of the eye (Ray and Schüpbach, 1996; Schweitzer and Shilo, 1997; Freeman, 1996); and Torso, which is involved in embryonic development (Duffy and Perrimon, 1994). Although each pathway uses a different RTK, all three employ the highly conserved Ras/Raf/MEK/MAPK cassette of proteins to reach their nuclear targets. Activation of each receptor triggers a relay of signals, first through the adaptor protein Drk, then the guanine exchange factor Sos, which activates Ras1, which in turn results in the sequential phosphorylation of the serine/threonine kinases Raf-1, MEK and MAP kinase (MAPK). MAPK then transmits the signal into the nucleus by phosphorylating a variety of nuclear proteins, including transcription factors.

Genetic evidence has suggested that activation of any of these three *Drosophila* RTKs may result in signaling through additional pathways. For example, a Ras-independent route to Raf activation from Torso has been demonstrated (Hou et al., 1995), and this has been confirmed by a biochemical study in mammals that implicates PLC-γ in such a role (Huang et al., 1995a). Using clones of mutant tissue in an otherwise wild-type *Drosophila* wing, another study showed that removal of Drk or Sos function has a less severe effect on DER-mediated phenotypes than removal of the receptor itself (Diaz-Benjumea and Hafen, 1994), again implying that multiple pathways are activated by DER.

Because it is activated by RTKs, mutations of PLC-γ might have been expected to appear in the plethora of large-scale genetic screens for modifiers of RTK signaling that have been conducted in both *Drosophila* and *Caenorhabditis elegans*. Isolation of PLC-γ mutations in either of these highly tractable invertebrate genetic systems would be extremely useful to open a window onto PLC-γ function in vivo; however none have been revealed until now. Here we show that the *Drosophila small wing* (*sl*) gene, originally identified by Bridges in 1915 (Morgan et al., 1925; Sivertzev-Dobzhansky and Dobzhansky, 1933), encodes a PLC-γ. Furthermore, we demonstrate that mutations of *sl* result not only in wing defects, but also in the development of extra R7 photoreceptors. Our data suggest that the eye phenotype results from overactivation of the Ras/Raf/MEK/MAPK cassette, implying that the PLC-γ encoded by *sl* normally acts as a negative regulator of Ras-mediated signaling during photoreceptor development in vivo.

MATERIALS AND METHODS

Drosophila stocks

sl^1 was obtained from a stock of fu^1/ClB (*ClB = In(1)Cl, sc l(1)C* t^2 *v* sl^1 *B*) and sl^2 from a stock of *In(1)bb*^{def} *y* $sl^2/FM4$, both from the *Drosophila* Stock Center at Indiana University. sl^3 ($Tp(1,2)r^{+75c}$) was obtained from A. Schalet (Yale University). Lines containing sl^1 and sl^2 mutations and no other visible markers were obtained by recombination using a *g sd f* chromosome. A deficiency for the *sl* locus, *Df(1)4b18/FM7a*, was kindly provided by R. Stanewsky (Brandeis University); the deficiency was generated on a *y cv v f car* background; a line free of these markers was obtained by recombination with a laboratory CS strain. The *sev sl* double mutants were made by recombination of *sl* alleles with *w* sev^1 or with *w* sev^{d2}; *PMC48/TM3*. The *sl*; *rl* double mutants were made by crosses using *M(1)15D/FM6*; *Sco/CyO*. The *sl*; *sina* double mutants were made by crosses to *C(1)M4 Y/FM7a*; *Sb/TM6*. The genotypes of all double mutants were confirmed by back-crosses to the parental lines. In the case of the back-crosses to the *sev* parental lines, the *sev* phenotype was confirmed by histological analysis. The *sev, flb* and *rl* alleles were obtained from the *Drosophila* Stock Center at Indiana University; a *st* $sina^2/TM3$ strain was provided by the Rubin laboratory (UC Berkeley).

Isolation and characterization of *Drosophila* PLC-γ

RNA was extracted from a crude mass-isolated antennal preparation and cDNA synthesized as described (Raha and Carlson, 1994). Degenerate primers corresponding to two conserved parts of region X (peptide SSHNTYL: CGGATCCKSNTCNCAYAAYACNTAYYT and peptide CVELDCW: CGAATTCCCARCARTCNARYTCNA-YRCA) were used in a PCR with parameters of 6 seconds at 96°C, 2 minutes 30 seconds at 45°C and 1 minute at 72°C, for 33 cycles. A single 3′ dA nucleotide overhang was added to the PCR product using Taq DNA polymerase and the product ligated into *Hinc*II-digested pBluescript II. One 126 bp clone had a sequence that closely matched that of mammalian PLC-γ isozymes. This clone hybridized to a single band of 2.1 kb when used to probe *Eco*RI-digested Southern blots of *Drosophila* genomic DNA. Primers directed against sequences within the clone were used in an inverse PCR reaction to amplify a 2.1 kb product from *Eco*RI-digested *Drosophila* genomic DNA; this product was then ligated into *Eco*RI-digested pBluescript. This clone was used to screen a *Drosophila* head cDNA library (T. Schwarz, Stanford University). The sequence of one cDNA, S3, matched the original 126 bp clone derived by degenerate PCR amplification. In situ hybridization to polytene chromosomes was performed as described by Ashburner (1989) using a biotinylated probe from the 2.1 kb genomic PLC-γ clone and immunoperoxidase detection.

Identification of molecular lesions in *sl* alleles

Southern blots of genomic DNA from all three *sl* mutants were probed with genomic and cDNA clones corresponding to the entire PLC-γ ORF. In sl^1, an insertion of 7.5 kb that matched both the size and restriction map described for the retrotransposon *412* (Lindsley and Zimm, 1992) was observed. The identity of the *412* element and the exact site of insertion was confirmed by PCR with a primer corresponding to the 3′ end of *412* (GTTAGAGGTAGCATATGCTC) and a primer from PLC-γ (AGTTCAGTGCGATCATCTGG). The 1.2 kb PCR product was digested with *Apa*I-*Sma*I, subcloned into pBluescript II and sequenced.

The genomic map around the PLC-γ ORF in sl^2 was identical to that of our laboratory Canton-S wild-type strain. To identify small alterations, total RNA was prepared from both wild-type and sl^2 homozygous adults, cDNA generated as described (Thackeray and Ganetzky, 1994) and two PCR amplifications performed. The first covered the 5′ half (1.9 kb) of the PLC-γ ORF, using primer 1 (GTTTGTATGCATTGCACTTA) and primer 2 (CCTTTCTGCCA-CCTTCAAGT, which crosses an intron/exon boundary and is expected to amplify only from cDNA) and the second covered the 3′ half (2.1kb) using primer 3 (GAAAACTTGAAGGTGGCAGA, which also crosses an exon/intron boundary) and primer 4 (ATACGTTTCAGATCCTAAGC). The product from each reaction

was digested with *Taq*αI and separated by electrophoresis in a 2.0% agarose gel; products from wild-type and mutant cDNA were loaded in adjacent lanes of the gel. A region containing a putative deletion in sl^2 was amplified afresh from both wild-type and sl^2 cDNA, using a different primer pair (primer 2 and primer 5: CAGGCCTTGATCGAGGTTTTCG) that gives a ~500 bp product. The reaction products were digested with *Bam*HI and compared by electrophoresis as described above, confirming the presence of a small deletion in sl^2. The *Bam*HI-digested fragment containing the deletion in sl^2 was subcloned into pBluescript and sequenced to determine its exact size and location.

The positioning of the distal breakpoint of $Tp(1;2)r^{+75c}$ within a 1.8 kb *Bam*HI fragment, previously determined by Jones and Rubin (1990), was confirmed and refined to within a 1.1 kb *Bam*HI-*Pst*I fragment by genomic Southern analysis.

Poly(A)$^+$ RNA was isolated from wild-type and *sl* adults, fractionated on a 1.0% agarose-formaldehyde gel and transferred to a Hybond-N+ (Amersham) nylon membrane. PLC-γ transcripts were detected by hybridization of the membrane with two non-overlapping PLC-γ cDNA probes. The same blot was reprobed with a fragment from the *Drosophila* actin 5C gene to determine the relative amount of RNA loaded in each lane.

Histological and immunohistochemical analysis

Adult heads were fixed, embedded in plastic, sectioned and stained with toluidine blue as described (Tomlinson and Ready, 1987). The R7-specific *Rh4-lacZ* fusion in P[*Rh4.1900 lacZ*] line 5 (homozygous for single insertions on both the second and third chromosomes; kindly provided by the Rubin laboratory, UC-Berkeley) was crossed into an sl^1 background. Heads were fixed, retinae dissected and stained with α-βgal monoclonal antibody 40-1a (obtained from the Developmental Studies Hybridoma Bank, University of Iowa) before being embedded in plastic. 2 μm sections were examined using Nomarski optics. Pupal eye imaginal discs, at 40 hours after puparium formation at 25°C, were stained with 'cobalt sulfide' as described by Cagan and Ready (1989).

RESULTS

small wing encodes a *Drosophila* PLC-γ

During a search for new PLC isozymes that might be involved in olfactory signal transduction (Riesgo-Escovar et al., 1995), we isolated a clone corresponding to a *Drosophila* PLC-γ homolog by PCR amplification from antennal RNA. After we had mapped the gene to cytological position 14B15 on the X chromosome and begun to characterize cDNA and genomic clones, a *Drosophila* PLC-γ homolog of identical sequence that mapped to the same cytological location was independently reported by another group (Emori et al., 1994). Our genomic restriction map of the PLC-γ locus allowed us to determine the exact location of the PLC-γ open reading frame (ORF) within two previously described chromosome walks on the X chromosome around *no-on-transient A* (*nonA*) at 14C1 (Jones and Rubin, 1990; Stanewsky et al., 1993; Fig. 1A). Moreover, one of these studies (Jones and Rubin, 1990) identified within this region the distal breakpoint of a chromosomal aberration, $Tp(1;2)r^{+75c}$, with which an allele of *small wing* (sl^3) has been associated (Craymer and Roy, 1980; Schalet, 1986). Because this breakpoint occurred within the proposed PLC-γ ORF, we suspected that the PLC-γ might be encoded by *sl*.

To investigate this possibility further, we examined the other two extant alleles of *sl* for molecular lesions in or around the PLC-γ ORF. We found that sl^1 has a 7.5 kb insertion of the retrotransposon *412* (Lindsley and Zimm, 1992) 3′ of nucleotide 3121 of the published sequence (Emori et al., 1994) and sl^2 has a 13 bp deletion, beginning 3′ of nucleotide 2027, that creates a stop codon beginning at nucleotide 2090 (Fig. 1B).

To confirm the identification of *sl* as the PLC-γ, we probed a northern blot of poly(A)+ RNA from wild-type and *sl* mutants with two non-overlapping PLC-γ cDNAs (Fig. 2). In lanes containing wild-type RNA, both probes hybridized primarily to a 6.2 kb transcript. In sl^1 adults, the 6.2 kb transcript is replaced primarily by a smaller product of 3.8 kb, presumably arising as a result of premature termination of transcription within the *412* element. sl^2 adults express a

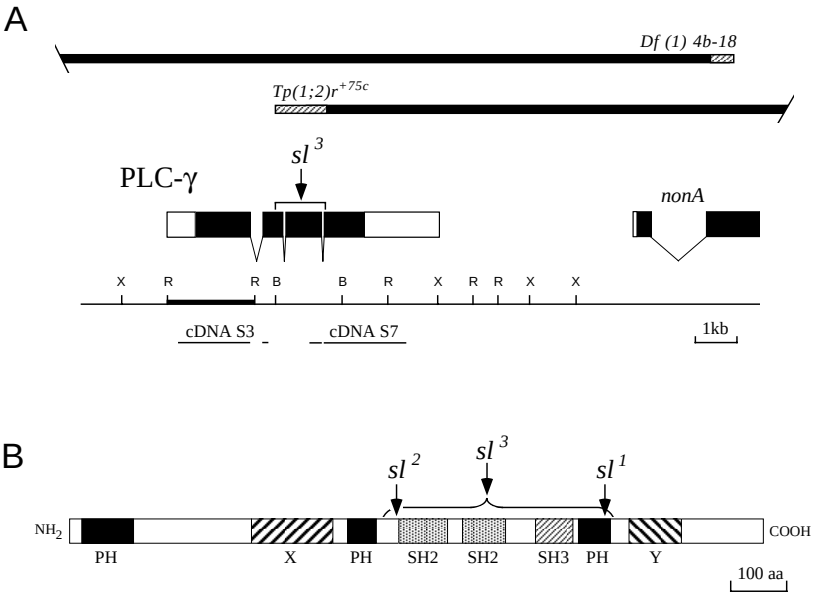

Fig. 1. Molecular characterization of the PLC-γ region. (A) Genomic restriction map of the PLC-γ region. The PLC-γ transcription unit is shown, with exons indicated by boxes (filled boxes represent translated sequences) and introns by lines connecting the boxes; the 5′ end of the transcript has not been determined precisely. Intron/exon structure is as described by Emori et al. (1994). The 2.1 kb *Eco*RI fragment cloned by inverse PCR is indicated by a heavy bar at the left end of the restriction map. The regions deleted by *Df(1)4b18* (Stanewsky et al., 1993) and transposed to chromosome 2 in $Tp(1;2)r^{+75c}$ (Jones and Rubin, 1990) are indicated by thick horizontal bars; the hatched box in each bar defines the limits within which the rearrangement breakpoints lie (Jones and Rubin, 1990; Stanewsky et al., 1993; this work). The positions of two PLC-γ cDNAs used as probes in Fig. 2 are shown. The organization of the *nonA* locus is as described elsewhere (Jones and Rubin, 1990; Stanewsky et al., 1993). B, *Bam*HI; R, *Eco*RI; X, *Xho*I. (B) Position of *sl* mutations within the PLC-γ open reading frame. The location of the X and Y catalytic domains in addition to the SH2, SH3 and PH domains within the deduced *Drosophila* PLC-γ protein are shown by shaded boxes. The positions of the *412* retrotransposon insertion in sl^1 and the 13 bp deletion in sl^2 are shown by arrows; for sl^3, the region within which the distal breakpoint of $Tp(1;2)r^{+75c}$ resides is indicated by a brace.

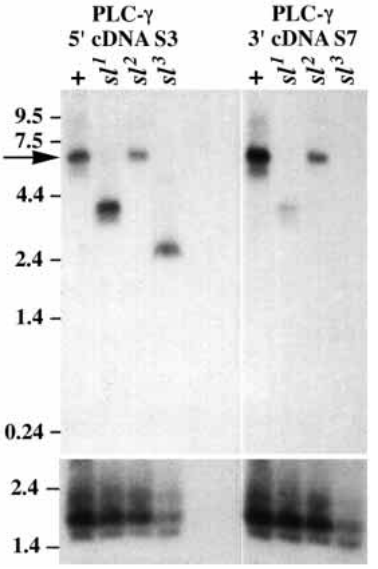

Fig. 2. Detection of PLC-γ transcripts in wild-type and *sl* mutants. The upper panel shows an autoradiogram of a pair of northern blots of poly(A)$^+$ RNA, each probed with one of two non-overlapping PLC-γ cDNAs. An arrow indicates the single 6.2 kb mRNA present in wild type. The lower panel shows part of the same blots reprobed with the *Drosophila* actin 5C gene to show the relative amount of RNA loaded in each lane. RNA molecular size standards for each panel are indicated on the left in kilobases.

transcript of normal size, but its abundance appears to be reduced, at least when probed with the more 3′ cDNA. This is consistent with the 13 bp deletion causing a frame shift, resulting in a premature stop codon and thereby inducing mRNA surveillance pathways that are thought to remove such aberrant transcripts (Beelman and Parker, 1995). sl^3, the allele associated with the $Tp(1;2)r^{+75c}$ breakpoint, displays a truncated transcript of 2.5 kb when probed with the 5′ cDNA, presumably corresponding to the length of the part of the gene remaining on the X chromosome. When probed with the 3′ cDNA, which corresponds to sequences transposed onto chromosome 2 and therefore separated from the *sl* promoter (Fig. 1A,B), no hybridization is visible in sl^3.

In summary, all three extant *sl* alleles have both a molecular lesion within the PLC-γ ORF and an altered transcript. On the basis of these molecular data and the reasonable agreement of the cytogenetic map positions of *sl* [estimated as approximately 14B13 (Schalet, 1986)] and PLC-γ (14B15), we conclude that *sl* encodes a *Drosophila* PLC-γ.

sl mutations affect both eye and wing development

All three *sl* alleles are recessive, homozygous viable mutations that are classically described as having a modest reduction in wing length and a mildly ‘rough’ eye (disturbance to the crystalline array of facets in the compound eye) (Lindsley and Zimm, 1992). In addition to these phenotypes, we found that animals homozygous for any of the three *sl* alleles have ectopic wing veins, most frequently adjacent to vein L2 (Fig. 3B,C, arrowheads), but sometimes also connected or adjacent to the posterior crossvein or within the posterior cell (Fig. 3C, upper and lower arrows, respectively). Heteroallelic combinations of all three alleles failed to complement with respect to the rough eye or wing phenotypes (data not shown).

This combination of ectopic wing veins and rough eyes resembles defects previously observed in mutations that activate the Ras/MAPK cassette, such as the *Ellipse* alleles of *DER* (DER^{ElpB1}) (Baker and Rubin, 1992), the *Sevenmaker* alleles of *rolled* (rl^{Sem}) (Brunner et al., 1994) and mutations of *Gap1* (Buckles et al., 1992; Gaul et al., 1992; Rogge et al., 1992). Because these mutations disrupt photoreceptor cell fate, we examined the compound eye from each of the *sl* alleles for photoreceptor defects that might underlie the observed roughness in the eye.

sl mutant eyes contain extra R7 photoreceptor cells

The wild-type *Drosophila* compound eye consists of about 800 hexagonal units, or ommatidia, packed in a crystalline array (Fig. 3D,E). In flies homozygous for any of the three *sl* alleles, this regular array is disrupted and many ommatidia are abnormally shaped (Fig. 3F,G). In addition, the *sl* eyes have

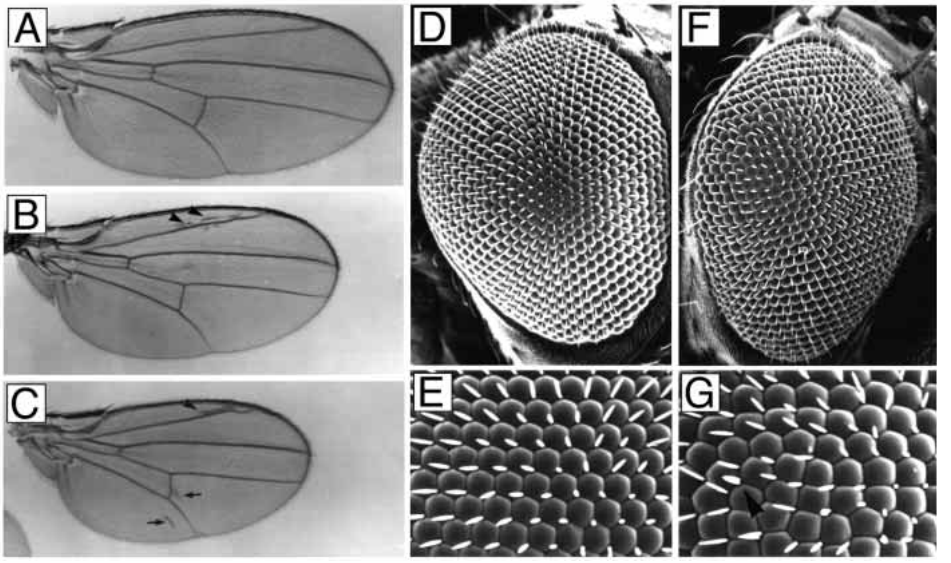

Fig. 3. Eye and wing phenotypes in mutant alleles of *sl*. Wings from (A) a wild-type female (Canton-S), (B) an sl^1 female and (C) an sl^3 male; all were photographed at the same magnification. Arrowheads in B and C indicate ectopic wing vein material between vein L2 and the wing margin; arrows in C indicate additional ectopic veins near the posterior crossvein (upper arrow) and also within the posterior cell (lower arrow). Scanning electron micrographs (SEM) of (D,E) wild-type and (F,G) sl^1 eyes; high magnification SEM views of wild-type (E) and sl^1 (G) demonstrate both the disordered packing of ommatidia and the duplication of some interommatidial bristles in *sl* mutants (indicated in G by an arrowhead). sl^2 and sl^3 have eye phenotypes indistinguishable from those seen in F and G (not shown).

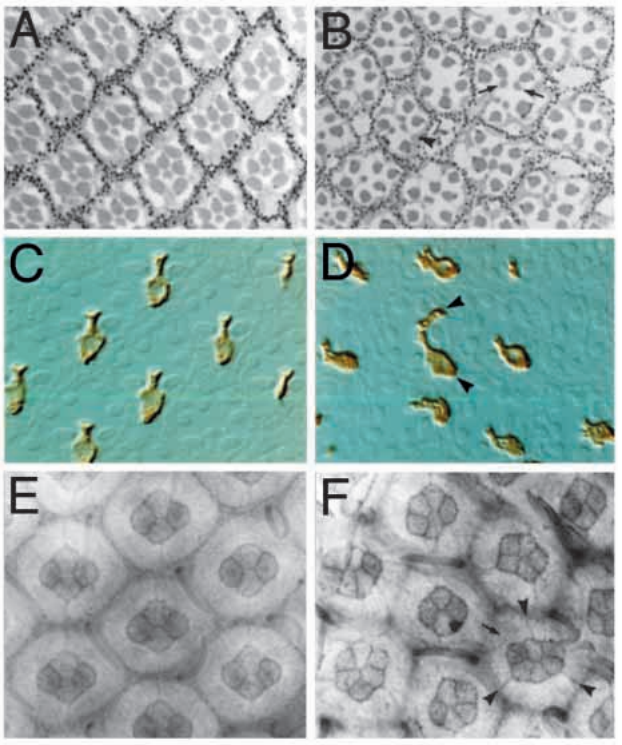

Fig. 4. Further characterization of defects in *sl* mutant eyes. Tangential sections through the distal part of eyes from (A) wild type and (B) sl^1 are shown, stained with toluidine blue. An sl^1 ommatidium containing three extra photoreceptor cells with small, centrally-projecting rhabdomeres is indicated by arrows in B. In addition, an ommatidium lacking the normal number of outer cells in sl^1 is indicated by an arrowhead in B. Sections through (C) wild type and (D) sl^1 adult retinae, each expressing the R7-specific *Rh4-lacZ* construct, stained with an anti-βgal antibody. An ommatidium with two cells staining with anti-βgal in the sl^1 retina is indicated with arrowheads in D, identifying the extra central photoreceptor as an R7 cell. Pupal eye discs from (E) wild-type and (F) sl^1 stained with cobalt sulfide (Cagan and Ready, 1989). Four cone cells surrounded by two primary pigment cells are seen in each ommatidium in the wild-type disc (E), whereas many ommatidia in the sl^1 disc (F) have one to three extra cone cells and/or an extra pigment cell. An arrow points to an ommatidium with six cone cells and more than two primary pigment cells; the arrowheads indicate pigment cell boundaries.

interommatidial bristle defects: some bristles are duplicated (Fig 3G, arrowhead), some are missing and some appear at additional vertices.

Each wild-type ommatidium contains a precise assembly of eight photoreceptor cells (R1-R8) capped by four lens-secreting cone cells and surrounded by a sheath of pigment and bristle cells (Wolff and Ready, 1993). The rhabdomeres (photosensitive organelles) of each of the six outer photoreceptor cells (R1-R6) can be seen in tangential sections to be arranged in a trapezoidal ring around a central, smaller rhabdomere (e.g. Fig. 4A). In the distal part of the retina, the central rhabdomere corresponds to photoreceptor R7; in more proximal parts, it corresponds to the R8 cell (Wolff and Ready, 1993). Tangential sections through the distal part of sl^1 homozygous mutant eyes indicated that many ommatidia

Table 1. Eye phenotypes of *sl* alleles and *sl* double mutants

Genotype	Wild-type* (%)	Extra R7‡ (%)	Missing R7 (%)	Extra R1-R6§ (%)	Missing ≥1 R1-R6 (%)
+	100	0	0	0	0
sl^1/+	100	0	0	0	0
sl^1	43±12	51±11	0	2±0.5	10±4
sl^2	61±5	37±5	0	2±0.8	3±0.7
sl^3	47±4	47±4	0	2±1	5±1
sl^1; flb^{1K35}/+	98±1	2±1	0	nd	nd
sl^2; flb^{1K35}/+	99±1	1±1	0	nd	nd
sl^1; flb^{1P02}/+	99±1	1±1	0	nd	nd
sl^2; flb^{1P02}/+	99±1	1±1	0	nd	nd
sev^1	0	0	100	0	0
sev^1sl^1	35±4	6±1	55±4	0	5±1
$sev^{d2}sl^1$	35±4	7±4	57±7	<1	<1
$sev^{d2}sl^2$	49±5	12±5	37±7	<1	5±1
rl^1	65±4	<1	22±2	0	15±4
sl^1; rl^1	57±8	<1	36±8	1±0.4	14±3
sl^2; rl^1	68±4	<1	27±3	1±0.4	10±2
$sina^2$	3±1	0	93±1	0	37±7
sl^1; $sina^2$	3±1	0	92±2	0	0
sl^2; $sina^2$	2±1	0	96±2	0	0

The percentages of ommatidia in each category were calculated from at least four separate heads, with between 40 and 120 ommatidia analyzed per eye, one eye per head. Some ommatidia fit in more than one category, so the figures do not sum to 100% for all genotypes. Experimental error is expressed in terms of the standard error of the mean.

*For both R7 and R1-R6 cells.

‡Identification of extra cells as R7 was confirmed with *Rh4-lacZ* only in the case of sl^1.

§Extra cells are labeled as R1-R6 based on rhabdomere size and position.

(51%) contain extra inner photoreceptors (i.e. with centrally located rhabdomeres) (Fig. 4B, arrows; Table 1). In addition, a small fraction of sl^1 ommatidia have one or more extra (2%) or missing (10%) outer photoreceptors. Similar phenotypes were observed in sl^2 and sl^3 eyes (Table 1).

The distal position, central location and small rhabdomere size of the extra inner photoreceptors in the *sl* mutants suggested that they were R7 cells. To confirm their identity, we examined the expression of an *Rh4-lacZ* fusion construct (Fortini and Rubin, 1990) in a sl^1 mutant background. In wild-type, expression of the Rh4 opsin is limited to a random subset (~70%) of R7 cells (Fortini and Rubin, 1990) (Fig. 4C). In sl^1 eyes, the majority of the supernumerary cells express the *Rh4-lacZ* gene (Fig. 4D, arrowheads), demonstrating that they have both the molecular and morphological characteristics of R7 photoreceptors.

In wild-type, the presumptive R7 cell is recruited from a pool of five *sevenless* (*sev*)-expressing cells known as the R7 equivalence group (Krämer and Cagan, 1994). After the R7 cell is recruited, the remaining four cells adopt a cone cell fate. The arrangement of the cone cells and the surrounding primary pigment cells can be seen in pupal eye imaginal discs by cobalt sulfide staining (Fig. 4E). sl^1 and sl^2 homozygotes contain one, two or sometimes as many as three more cone cells than wild-type (Fig. 4F); in addition, an extra primary pigment cell is sometimes present. Again, these phenotypes are remarkably similar to those seen in mutations that increase the number of R7 cells, such as sev^{S11} (Basler and Hafen, 1989), rl^{Sem} (Brunner et al., 1994), *Gap1* (Buckles et al., 1992; Gaul et al., 1992; Rogge et al., 1992) and *yan* (Lai and Rubin, 1992).

Genetic analysis confirms that the *sl*^{1} and *sl*^{2} mutations are null

The nature and position of the molecular defects in all three *sl* alleles would suggest that they are likely to be null, because one of the two catalytic domains found in all phospholipase C proteins described to date, region Y, is predicted to be missing from any protein product. We used a deficiency chromosome that uncovers *sl*, *Df(1)4b18* (Stanewsky et al., 1993) to determine whether *sl*^{1} and *sl*^{2} are null by genetic criteria. If either mutation caused only a partial loss of function, the phenotype when heterozygous with a deficiency (*sl*/*Df*) would be more severe than that of a homozygote (*sl*/*sl*). The frequency of ommatidia with extra R7 cells of either allele in heterozygous combination with *Df(1)4b18* (24.3±6.0% and 22.1±5.9% for *sl*^{1}/*Df* and *sl*^{2}/*Df*, respectively) was indistinguishable from the homozygous phenotype of the parents (26.5±6.4% and 32.5±3.9% for *sl*/*sl1*^{1} and *sl*^{2}/*sl*^{2} respectively) in an experiment that compared them directly. The wing length of the homozygotes is also indistinguishable from the wing length of either allele heterozygous with the same deficiency (Fig. 5A), at about 80% of the wild-type length. The *sl*^{1} and *sl*^{2} mutations are therefore behaving as expected for null alleles for both the eye and wing phenotypes.

To rule out the possibility of any gain-of-function effect from the mutations, we examined the effect of adding a copy of *sl*$^{+}$ into a homozygous mutant background using a duplication that includes the *sl* region, *Dp(1;4)r*$^{+}$ (Lindsley and Zimm, 1992). A single copy of *sl*$^{+}$ in animals homozygous for either *sl*^{1} or *sl*^{2} was able to rescue completely both the eye (100% of the ommatidia had one R7 cell; *n*=7, 50 ommatidia per animal) and wing phenotypes (Fig. 5B). This result is not consistent with a gain-of-function model, because a novel activity produced by a mutant allele would be unaffected by the presence of the wild-type gene. It is, however, completely consistent with a loss-of-function model: the lost *sl* activities in the *sl*^{1} and *sl*^{2} homozygotes are provided by the wild-type copy of *sl* on the duplication chromosome. Taken together, the genetic evidence therefore agrees with the molecular data, indicating that both *sl* alleles are null mutations.

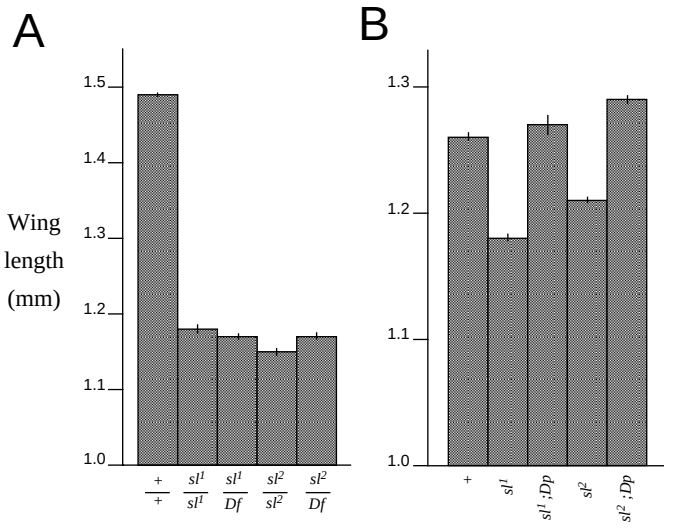

Fig. 5. Wing lengths of *sl* in combination with deficiencies or duplications of the *sl* region. (A) Wing lengths of *sl* homozygotes compared both with wild-type and in sl/*Df(1)4b18*. The numbers of animals scored (one wing per animal) for each genotype were: +/+ (Canton-S), *n*=39; *sl*^{1}, *n*=28; *sl*^{1}/Df, *n*=15; *sl*^{2}, *n*=23; *sl*^{2}/Df, *n*=16. (B) Wing lengths of *sl* mutants with or without a single copy of *Dp(1;4)r*$^{+}$. For +/+ (Canton-S), *n*=39; for all other genotypes, *n*=10-15. All flies in B also carried *forked (f)* to allow flies either carrying the duplication (which are *f*$^{+}$ because the duplication includes *f*$^{+}$ as well as *sl*$^{+}$) or lacking the duplication (which are *f*) to be distinguished. The flies for each *sl* allele are siblings from the same cross. Wing length for each fly was determined by measuring from the point where the anterior crossvein intersects the third longitudinal vein, to the point where the same longitudinal vein meets the wing margin. The s.e.m. for each genotype is represented by a vertical line on each bar.

Sl acts as a negative regulator that acts upstream of MAPK

To determine whether the effect of the *sl* mutations on cell fate determination in the eye is via the Ras/MAPK pathway, we made a series of double mutants. Signaling via the Ras/MAPK cascade occurs in the developing R7 cell from at least two RTKs, Sev and DER. We asked whether *sl* affects DER signaling, by examining the effect of reduced *DER* dosage in a background that was homozygous for either *sl*^{1} or *sl*^{2}. Reducing the dosage of *DER* using either of two independent null mutations of *DER*, *flb*^{1k35} and *flb*^{1P02}, in either an *sl*^{1} or *sl*^{2} background, almost completely rescued the eye phenotype (Fig. 6C,D; Table 1). For example, whereas approximately half of the ommatidia contained an extra R7 cell in *sl*^{1}; +/+ males, this was reduced to 2% of ommatidia in *sl*^{1}; +/*flb*^{1k35}. This result shows that the supernumerary R7 cells seen in the *sl* mutants depend on DER; even a halving of *DER*$^{+}$ dosage is sufficient to suppress the eye phenotype almost completely.

We also asked whether *sl* affects Sev signaling. Null mutations of *sev* result in the loss of the R7 cell from all ommatidia (Tomlinson and Ready, 1986, 1987). Double mutants of *sl*^{1} or *sl*^{2} with either *sev*^{1} or *sev*^{d2} (a null allele) had an eye phenotype that was intermediate between the two single mutants – 35-49% of ommatidia had one R7 cell, an additional 6-12% contained two R7 cells and the remaining ommatidia lacked R7 cells (Table 1; Fig. 6F). This partial suppression of the *sl* mutant phenotype shows that production of the extra R7 cells in the *sl* homozygotes is not completely dependent on *sev*$^{+}$ activity, and confirms the result of Freeman (1996) that R7 cells can be produced in a *sev*-independent manner.

To determine whether there are interactions between *sl* and other genes of the Ras pathway, we initially used a mutation of MAP kinase, *rolled (rl*^{1}*)*. This mutation is viable and thus its effects can easily be examined in the adult eye (homozygous loss-of-function mutations of most known components acting downstream of the Sev and DER RTKs are embryonic lethal). The partial loss-of-function mutation of MAPK, *rl*^{1}, has a mild impact on R7 formation: 22% of the ommatidia lack R7 cells (Biggs et al., 1994; also Table 1; Fig. 6G). Both *sl*^{1}; *rl*^{1} and *sl*^{2}; *rl*^{1} double mutants have phenotypes comparable to the *rl*^{1} single mutant: 36% and 27%, respectively, are missing R7 cells and <1% contain extra R7 cells (Table 1, Fig. 6H). Thus, whereas in *sl*^{1} or *sl*^{2} mutants 51% and 37%, respectively, of ommatidia have extra R7 cells, this is reduced to <1% in a *rl*^{1} background.

The phenotype of the *sl;rl* double mutants suggests that *sl* is acting upstream of *rl*. To confirm this result, we tested for

interaction between *sl* and a gene downstream of *rl*, *seven in absentia* (*sina*), which encodes a nuclear protein (Carthew and Rubin, 1990) and for which a viable loss-of-function allele, $sina^2$, is also available. In $sina^2$, the proportion of ommatidia with an R7 cell is less than 5% (Carthew and Rubin, 1990; also Table 1; Fig. 6I). Adding either sl^1 or sl^2 into a $sina^2$ background caused no increase in the proportion of ommatidia with R7 cells (Table 1, Fig. 6J). Thus the results with *sina* are consistent with those with *rl*, indicating that *sl* affects Ras signaling upstream from the *rl* MAP kinase.

The wing length phenotype observed in the *sl* single mutants is unaffected in any of the homozygous double mutant combinations (data not shown). In contrast, the ectopic wing vein phenotypes of sl^1 and sl^2 are strongly suppressed by rl^1: in sl^1, 49% of wings (n=43) have ectopic veins, compared to only 7% (n=41) in the double mutant sl^1; rl^1 (Fig. 7B,D).

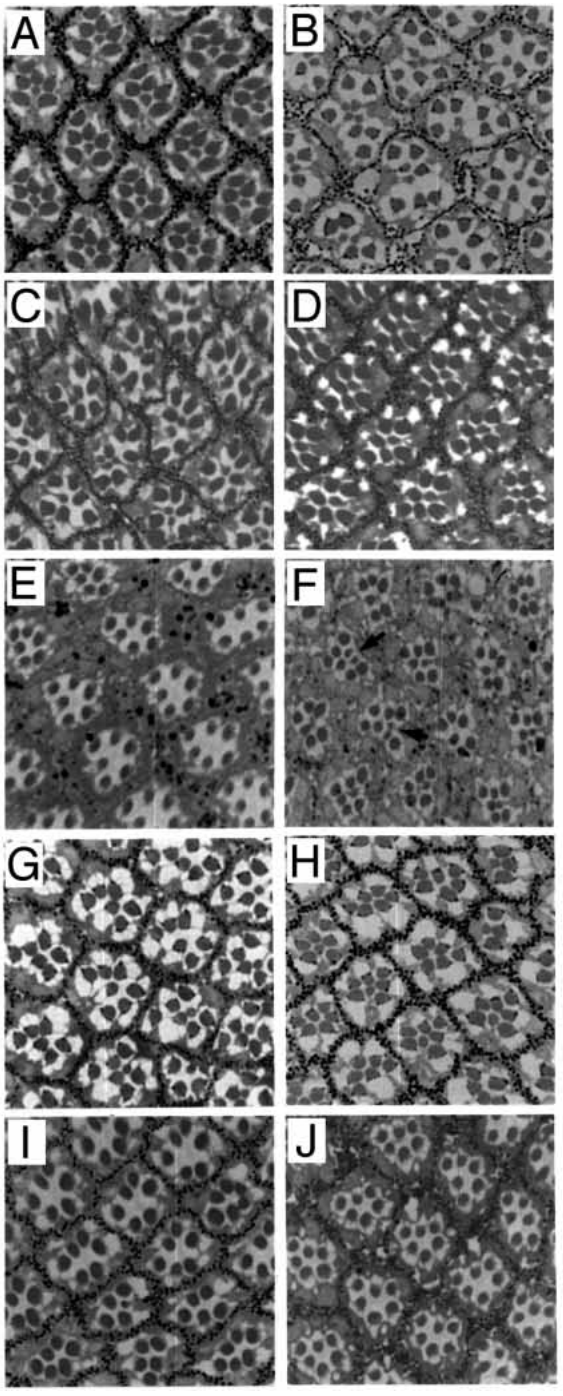

Fig. 6. Eye phenotypes of double mutant combinations of *sl* with mutants in the Sevenless/DER pathways. Tangential sections of eyes from (A) wild-type, (B) sl^1, (C) sl^1; +/+, (D) sl^1; +/flb^{1K35}, (E) *w* sev^1, (F) *w* sev^1 sl^1, (G) rl^1, (H) sl^1; rl^1, (I) $sina^2$, (J) sl^1; $sina^2$, stained with toluidine blue. The animals represented in C and D were siblings. The arrows in F indicate two ommatidia with an extra R7 cell.

DISCUSSION

We have shown that *sl*, identified by Bridges in 1915, encodes a PLC-γ. This conclusion is based on the following evidence: (i) all three extant *sl* alleles contain molecular lesions in the PLC-γ open reading frame that would each result in the loss of a critical catalytic domain; (ii) *sl* mutants contain altered PLC-γ transcripts and (iii) the map positions of *sl* and of the *Drosophila* PLC-γ gene are within reasonable agreement in cytogenetic region 14B on the X chromosome.

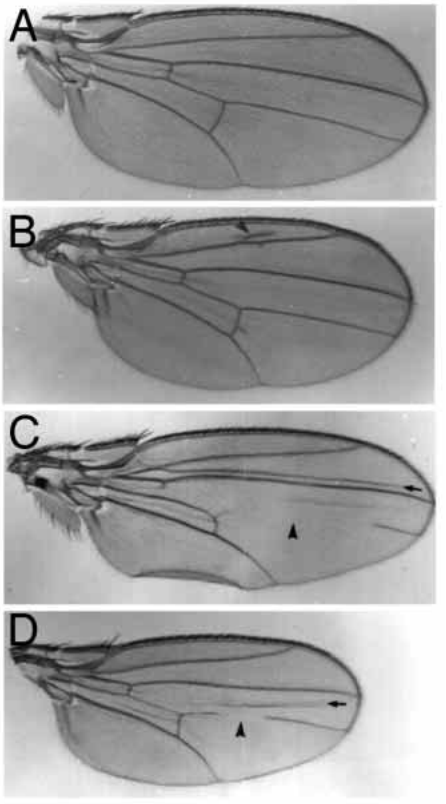

Fig. 7. Wing phenotypes of double mutant combinations of *sl* with mutants in the Sevenless/DER pathways. Wings from (A) wild-type (Canton S), (B) sl^1, (C) rl^1, and (D) sl^1; rl^1. The arrowhead in B indicates ectopic wing vein material beside vein L2 in an sl^1 wing. Note the absence of ectopic wing veins beside L2 in the sl^1; rl^1 double mutant (D). The arrows in C and D indicate the positions of longitudinal creases in the wing that appear in flies homozygous for rl^1, due to the lateral curling of the wing for which *rl* is named. The arrowheads in C and D indicate gaps in vein L4, a phenotype characteristic of *rl* (Lindsley and Zimm, 1992).

We have documented eye and wing defects in *sl* mutants, including extra R7 cells and ectopic wing veins. Similar eye and wing vein phenotypes have been shown to be caused by mutations that activate the Ras pathway. The phenotypes that we observed in double mutants of *sl* with various mutations in this pathway are consistent with a model in which *sl* mutations activate the Ras/MAPK pathway downstream of DER and upstream of the MAPK encoded by *rl*. This interpretation is based primarily on the findings that (1) the extra R7 cells seen in both sl^1 and sl^2 are dominantly suppressed by null alleles of *DER*, (2) in a background lacking any *sev* activity both sl^1 and sl^2 mutations result in the formation of R7 cells, (3) the formation of extra R7 cells in sl^1 or sl^2 depends completely on both *rl* and *sina* and (4) rl^1 suppresses the ectopic wing veins present in sl^1. Although the *sl* eye phenotype was partially suppressed in a *sev* null background, the intermediate phenotype produced can be explained as an additive effect, in which '*sev*-derived' R7 cells are lost and the remaining observed cells are produced by overactivation of DER. Overactivation of DER has previously been shown by Freeman (1996) to generate extra R7 cells, even in the complete absence of Sev function, in an experiment that expressed the DER ligand Spitz using a *sev*-enhancer. However, our results do not exclude the possibility that Sl may also act downstream of Sev.

These data suggest that the *sl*-PLC-γ normally has a role as a negative regulator in the pathways leading to R7 development. When this down-regulatory role is lost in the mutants, the increased and/or prolonged signal presumably results in additional cells being recruited to an R7 cell fate. If Sl is playing a negative role in R7 development, how might it be doing so? The principal catalytic function of PLC-γ is its hydrolysis of PIP_2 into DAG and IP_3. DAG is known to activate PKC (Nishizuka, 1995), which has been shown to have a role as an activator of RTK-signaling in some contexts, for example by phosphorylation of Raf (Sözeri et al., 1992; Kolch et al., 1993; Cai et al., 1997), and as an inhibitor in others (Decker, 1984; Takayama et al., 1988). Another study showed that sustained stimulation of PKCα by a phorbol ester in NIH3T3 cells led to an association of PKC with the EGF receptor, followed by phosphorylation of the RTK and subsequent internalization and/or degradation of the receptor (Seedorf et al., 1995). Interestingly, overexpression of PLC-γ was found to enhance this down-regulatory effect, consistent with a role for PLC-γ as a negative feedback regulator of signaling in this pathway. The results that we have described are consistent with such a role for Sl, acting as a direct or indirect inhibitor/attenuator of the DER signal. It may be that Sl is both activated by DER and then later required to attenuate the DER signal. When the DER signal is allowed to persist due to the absence of Sl activity, extra R7 cells are produced.

Most models of PLC-γ activation propose that one of its SH2 domains binds to specific phosphotyrosines on the activated RTK, as has been demonstrated experimentally for the PDGF receptor and PLC-γ1 (Koch et al., 1991). However, there is no consensus site for mammalian PLC-γ SH2 binding (YDTP/YDIP; Songyang et al., 1993) in the intracellular domain of DER. It may be that Sl does not bind directly to DER, or it might do so at a different sequence. If it does not bind to DER it is possible that it is activated by binding to another RTK, or that it interacts with another protein which is itself activated by DER. One such intermediary protein might be Daughter of sevenless (Dos), which is required for Sev signaling (Raabe et al., 1996; Herbst et al., 1996) and is proposed to act as an adaptor protein that brings together a multiprotein complex at an activated RTK (Raabe et al., 1996). The Dos sequence contains consensus sites for binding mammalian PLC-γ SH2 domains and a polyproline domain that might bind to the SH3 domain of PLC-γ (Schlessinger, 1994; Pawson, 1995).

Another protein that is likely to interact with Sl is the membrane protein PI3K. A recent biochemical study has shown that an adaptor protein for a *Drosophila* PI3K also binds to the *Drosophila* PLC-γ that we show here to be encoded by *sl* (Weinkove et al., 1997). This interaction might be involved in targeting Sl to the membrane, as has been demonstrated for mammalian PLC-γ (Falasca et al., 1998). Once at the membrane and activated by association with the RTK (presumably DER), PLC-γ induced activation of PKC could result in phosphorylation either of one or more components of the multiprotein complex assembled at the RTK, or the RTK itself – this phosphorylation being required to terminate the signal correctly. Whatever the mechanism, it will be of interest to see what interactions exists between PI3K, Dos and Sl.

The reduced wing-length phenotype observed in all three *sl* alleles could be due to a reduction in cell number, reduced cell size or both. It has previously been shown that, in wing tissue homozygous for mutations in any of six different genes that reduce signaling in the DER pathway, including *DER*, *drk*, *sos*, *Ras1*, *Raf* and *rl*, cell density is higher than in surrounding wild-type wing tissue (Diaz-Benjumea and Hafen, 1994). In addition, where such mutant clones overlap a vein, no vein is produced. This latter finding is consistent with our interpretation of Sl as a negative regulator of RTK signaling, in that the *sl* mutations result in the opposite phenotype: extra wing veins. The effect of the *sl* mutations on wing size appears to be mediated by a pathway different from that used in wing vein development, because the *sl* mutations in this case show a similar phenotype to those referred to above that reduce DER function. The fact that *rl* suppresses the extra wing vein phenotype but not the wing size phenotype of *sl*, is also consistent with a role for Sl in two different pathways governing wing development.

One intriguing aspect of the *sl* mutations is that they have such a minimal effect on viability; indeed, all three alleles can be maintained as homozygous stocks. By contrast, the recently identified mouse PLC-γ1 knockout (Ji et al., 1997) is a recessive lethal mutation, in which homozygotes die during early embryogenesis. It may be that, in *Drosophila*, there is redundancy within the signaling pathways in which PLC-γ is involved, provided either by another PLC-γ gene or from other components of these pathways. Such redundancy would also help to explain why PLC-γ mutations have not been isolated in the many large-scale screens for enhancers and suppressors of RTK signaling pathways in both *Drosophila* and *C. elegans*.

In summary, the analysis of *sl* presented here adds PLC-γ to an expanding list of players in Ras/MAP kinase signaling pathways in *Drosophila*. The availability of PLC-γ mutations now paves the way for an extensive genetic analysis of its role in RTK signaling pathways and the identification of other signaling components with which it interacts.

We take this opportunity to express our sadness at the passing of

Abe Schalet (1928-1997), whose encyclopedic knowledge of *Drosophila* genetics and frequent provision of critical stocks greatly facilitated this and many other studies by our laboratory. We thank K. Matthews, R. Stanewsky, M. Fortini, T. Laverty and G. Rubin for additional fly stocks, P. Clyne and J. Riesgo-Escovar for discussions and G. Fitzgerald for help with histology. Supported by research grant number RO1 DC02174 (to J. R. C.) from the National Institute on Deafness and Other Communication Disorders, NIH, and NIMH fellowship MH10572 (to P. G.). P. R. E. was a Monsanto Fellow of the Life Sciences Research Foundation.

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