Distinct Phospholipase C-γ-Dependent Signaling Pathways in the Drosophila Eye and Wing Are Revealed by a New *small wing* Allele

Rishikesh Mankidy, Jeremy Hastings and Justin R. Thackeray[1]

Department of Biology, Clark University, Worcester, Massachusetts 01610

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ABSTRACT

The Drosophila genome contains a single phospholipase C-γ (PLC-γ) homolog, encoded by *small wing* (*sl*), that acts as an inhibitor of receptor tyrosine kinase (RTK) signaling during photoreceptor R7 development. Although the existing *sl* alleles behave genetically as nulls, they may still produce truncated Sl products that could in theory still provide limited PLC-γ function. Both to identify a true null allele and to probe structure-function relationships in Sl, we carried out an F_1 screen for new *sl* mutations and identified seven new alleles. Flies homozygous for any of these alleles are viable, with the same short-wing phenotype described previously; however, two of the alleles differ from any of those previously isolated in the severity of the eye phenotype: sl^9 homozygotes have a slightly more extreme extra-R7 phenotype, whereas sl^7 homozygotes have an almost wild-type eye. We determined the mutant defect in all seven alleles, revealing that sl^9 is a molecular null due to a very early stop codon, while sl^7 has a missense mutation in the highly conserved Y catalytic domain. Together with *in vitro* mutagenesis of the residue affected by the sl^7 mutation, these results confirm the role of Sl in RTK signaling and provide evidence for two genetically separable PLC-γ-dependent pathways affecting the development of the eye and the wing.

PHOSPHOLIPASE C-γ (PLC-γ) is involved in regulating a diverse array of cellular processes, including proliferation, differentiation, and motility (reviewed by Rebecchi and Pentyala 2000). PLC-γ activation is triggered by the binding of a wide variety of growth factors, cytokines, and immunoglobulins to their membrane-bound receptor. The activated enzyme hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] into two intracellular second messengers: inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$], which increases intracellular [Ca^{2+}] by release from the endoplasmic reticulum, and diacylglycerol, which, in combination with Ca^{2+}, activates the classical isoforms of protein kinase C. More recently it has become clear that PLC-γ must be viewed as more than simply a phospholipase. One study showed that a PLC-γ protein lacking phospholipase activity was able to stimulate a partial mitogenic response (Smith *et al.* 1994), and another study showed that this ability depends on an intact SH3 domain (P. S. Huang *et al.* 1995). The PLC-γ SH3 domain has now been shown to bind to an enhancer of phosphatidylinositol-3-OH kinase [PI(3)K] called PI(3)K enhancer (PIKE), acting as a guanine nucleotide exchange factor for PIKE, and thereby augmenting nuclear PI(3)K activity (Ye *et al.* 2002). PLC-γ is therefore able to stimulate mitogenesis even when incapable of phospholipid hydrolysis.

[1]*Corresponding author:* Biology Department, Clark University, 950 Main St., Worcester, MA 01610. E-mail: jthackeray@clarku.edu

Two distinct but very similar PLC-γ isoforms have been described in mammals, PLC-γ_1 and PLC-γ_2, which are encoded by different genes. Both are widely expressed throughout development, but the γ_2 isoform is most abundant in cells derived by hematopoiesis (Homma *et al.* 1989). Mice homozygous for knockout mutations of PLC-γ_1 die during the early stages of embryogenesis (Ji *et al.* 1997), whereas PLC-γ_2 knockouts are viable but result in impaired B-cell development and a general failure of B-cell antigen receptor signaling (Hashimoto *et al.* 2000; Wang *et al.* 2000). Vertebrates have three other PLC types, PLC-β, PLC-δ, and PLC-ε, which differ from PLC-γ in overall structure, pattern of expression, and mode of activation, but are thought to have similar catalytic abilities (reviewed by Rhee 2001).

A single PLC-γ gene has been identified in Drosophila, encoded by *small wing* (*sl*) (Thackeray *et al.* 1998). This sequence is the only PLC-γ homolog present in the genome and is equally similar to mammalian PLC-γ_1 and PLC-γ_2 (Manning *et al.* 2003). Four mutant *sl* alleles have been recovered since its original discovery by Bridges in 1915. Three of these alleles are extant: sl^1, sl^2, and sl^3 are all recessive and homozygous viable alleles, with a slightly shortened wing and a mildly rough eye (Morgan *et al.* 1925; Sivertzev-Dobzhansky and Dobzhansky 1933; Schalet 1986). One additional allele, sl^{34}—isolated by Gottschewski in 1934 but now lost—was reported to differ from the other alleles in that it had shortened wings but normal eye morphology (Lindsley and Zimm 1992). More recently it was shown that $sl^{1,2,3}$ homozygotes have extra-R7 photoreceptor

cells in 30–50% of the ommatidia (unit eyes) of the compound eye, and their wings occasionally contain ectopic patches of wing-vein-like material (Thackeray *et al.* 1998). Assignment of cell fate in the developing eye and wing of Drosophila depends on the correct timing of a number of signals, especially those derived from the Notch, Sevenless, and Egfr signaling pathways (Diaz-Benjumea and Hafen 1994; Freeman 1997; Brennan and Moses 2000). In the case of the receptor tyrosine kinases (RTKs) Sev and Egfr, each passes a signal to the nucleus via the highly conserved Ras/Raf/mitogen-activated protein kinase (MAPK) "cassette" (reviewed by Tan and Kim 1999).

The mild effect of the *sl* alleles is surprising for a variety of reasons. First, null mutations in almost all the other members of the Ras/Raf/MAPK-mediated signaling pathways are lethal (Zipursky and Rubin 1994). Second, loss of mammalian PLC-γ_1, which probably has the most similar functional role to Sl among the two mammalian PLC-γ homologs, results in early embryonic lethality in mouse knockouts. One possible explanation of this puzzle is that PLC-γ is involved in mammalian RTK signaling both as a positive (J. Huang *et al.* 1995) and as a negative (Obermeier *et al.* 1996) regulator. However, the three extant *sl* alleles have molecular defects that would truncate Sl, but not necessarily eliminate all Sl functions (Thackeray *et al.* 1998); despite this, the sl^1 and sl^2 alleles both behaved genetically as nulls when made heterozygous with deficiencies or duplications for the region containing *sl* (Thackeray *et al.* 1998). Genetic epistasis experiments in the same study showed that Sl acts as a negative regulator upstream of MAPK and is most likely activated by Egfr. However, the shortened wing phenotype appears to be due to disturbance of a MAPK-independent pathway, because it is not suppressed by rl^1, a hypomorphic allele of the MAPK encoded by *rolled*. In contrast, rl^1 suppresses both the extra-R7 and ectopic wing-vein phenotypes completely (Thackeray *et al.* 1998).

Although the mechanism by which Sl inhibits RTK signaling is not yet determined, a strong positive genetic interaction between *sl* alleles and mutations of *GTPase activating protein 1* (*Gap1*; Powe *et al.* 1999) suggests that it may be via indirect downregulation of Ras1. Gap1 was first identified as a negative regulator of Ras-mediated signaling in the eye (Gaul *et al.* 1992), where it is presumed to promote conversion of active Ras-GTP to the inactive GDP-bound form. On the basis of the genetic interaction between *sl* and *Gap1* and other evidence, a model was proposed in which Gap1 is activated by the PLC-γ-induced rise in concentration of both inositol 1,3,4,5-tetrakisphosphate [I(1,3,4,5)P_4] and Ca^{2+}, resulting in the downregulation of Ras by the activation of its GTPase activity (Powe *et al.* 1999).

The "standard model" for mammalian PLC-γ activation involves binding of a src-homology-2 (SH2) domain in PLC-γ to the activated receptor, followed by phosphorylation of one or more tyrosine residues as a result of RTK activity or from a receptor-associated tyrosine kinase such as Src (reviewed by Rhee 2001). However, it has also been shown that PLC-γ can be activated without tyrosine phosphorylation by binding phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] to either the N-terminal pleckstrin homology (PH) domain (Falasca *et al.* 1998) or the C-terminal SH2 domain (Rameh *et al.* 1998). PLC-γ contains several additional motifs common to many signaling proteins: a second PH domain, an EF hand region, an N-terminal SH2 domain, one SH3 domain, and a C2 domain. Although some attempts have been made to dissect the roles of these domains in mammalian PLC-γ by *in vitro* mutagenesis (Chattopadhyay *et al.* 1999), such targeted approaches require that prior assumptions be made about the likely functional importance of particular amino acids. By contrast, a random mutagenesis *in vivo* can identify important residues purely on the basis of the phenotypic consequences of their loss. In this study we describe such a screen for new *sl* alleles, using ethyl methanesulfonate (EMS) mutagenesis. Seven alleles were isolated and characterized, revealing that the originally identified *sl* alleles may still retain some function and the phenotype of one allele demonstrates the presence of independently mutable pathways for PLC-γ signaling in the eye and wing.

MATERIALS AND METHODS

Fly stocks: Flies were raised on a standard cornmeal, molasses, yeast, agar medium at 25°. The *w* sl^1 stock was originally derived by recombination from a *ClB* balancer chromosome as described previously (Thackeray *et al.* 1998). The *M9/ClB* balancer stock was generated from the *ClB* stock described above, and an *M9* stock was provided by the late Abe Schalet (Yale University); *M9* is a version of *In(1)*sc^8 + *dl49* and carries the following markers: y^{31d} sc^8 w^a mei^{9L1} *vb* v^{0f} *f* (Lindsley and Zimm 1992). The *y cv v f* and *FM6/FM7c* stocks were originally obtained from the Indiana Stock Center.

EMS mutagenesis: *y cv v f* males were collected within 24 hr of eclosion and aged for 2–5 days on yeasted vials. After being allowed to feed on a solution of 0.2% EMS (Sigma, St. Louis) in 1% sucrose overnight, the males were placed on fresh standard food vials for 24 hr and then mated with virgin *w* sl^1 females. A line was established from each F_1 female carrying any combination of rough eyes, altered wing veination, or shortened wing by crossing to *M9* males. The male *forked* progeny (*f* lies 3 cM proximal to *sl*) in each line were rescored for *sl*-like phenotypes; if no *forked* males were recovered among the F_2, indicating a lethal hit at or near *sl*, virgin *v f* F_2 females (which will carry the mutagenized X chromosome balanced over *M9*) were backcrossed to *w* sl^1 males to allow hits at *sl* to be scored. Lines carrying a new *sl* mutation were outcrossed to wild type and all other visible X-linked markers were removed by standard recombination crosses before being balanced over *FM7c*. Histological analysis of wings and eyes was performed as described previously (Thackeray *et al.* 1998).

Determination of *sl* mutations: Genomic DNA was isolated from homozygous adults of each strain and the entire *sl* gene examined for mutations by single-strand conformational polymorphism (SSCP) essentially as described (Orita *et al.* 1989).

The gene was amplified in six overlapping PCR reactions, using the following primer pairs:

chunk 1: GN5/SG3 (5′ GTTTGTATGCATTGCACTTA 3′/5′ GGCAATGGCTGCGCTACTGA 3′, corresponding to positions 162–181 and 1094–1113 of the published *sl* sequence; Emori *et al.* 1994);
chunk 2: CP5/CP1 (5′ GCAGTTCCTCGAGACGGAAC 3′/5′ GTTGCGTAAGCAGCATATCC 3′, 1050–1590, includes an additional 358 bp of intron 1);
chunk 3: CP4/CP6 (5′ CCTGACGGGCGATCAGTTCTCCA 3′/5′ GGAGTTTCCGTTGCGTGACTCG 3′, 1302–1935, also includes intron 1);
chunk 4: CP7/CP3N (5′ ACTTCAAGGATCCGGTCGATAA 3′/5′ GGACATTCGTTATGATGGCATGC 3′, 1828–2849, includes 65 bp of intron 2);
chunk 5: CP2N/GYB3 (5′ CTTCGAGTCTCTGGTCTCGCTGA 3′/5′ AGTTCAGTGCGATCATCTGGGA 3′, 2565–3460, includes 59 bp of intron 3);
chunk 6: CP8/GH3N (5′ CTCCTACCATCGCAATCAGATT 3′/5′ TCATACGTTTCAGATCCTAAGCT 3′, 3345–4191).

These amplifications include the whole *sl* open reading frame (ORF) from 115 bp upstream of the initiating methionine to 204 bp downstream of the stop codon, as well as all three introns.

Each PCR reaction was carried out essentially as described (Thackeray *et al.* 1998), except that the reaction volume was 20 μl and contained 2 μCi [α^{32}-P]dATP. Parameters used were 94° for 45 sec, 56° for 45 sec, and 72° for 2 min for 36 cycles. An aliquot of each reaction was then digested in two different single-enzyme digests in a 15-μl volume as follows: chunk 1 with *Alu*I and *Dpn*II, chunk 2 with *Taq*I and *Hpa*II, chunk 3 with *Taq*I and *Hha*I, chunk 4 with *Alu*I and *Fok*I, chunk 5 with *Taq*I and *Hpa*II, and chunk 6 with *Alu*I and *Hin*fI. Stop solution (10 μl 95% formamide, 20 mm EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added, the sample was denatured at 100° for 2 min, and 3 μl was separated on a nondenaturing mutation detection enhancement acrylamide gel according to the manufacturer's instructions for standard SSCP (Cambrex, East Rutherford, NJ). After autoradiography, banding patterns between *sl* mutants and the *y cv v f* parental line were compared; a single region with altered mobility compared to the parental line was found for each mutant allele. The PCR reaction containing the band with altered mobility was repeated from a fresh genomic DNA sample and sequenced without subcloning using the fmol sequencing kit (Promega, Madison, WI) with internal primers predicted to be close to the site of mutation. A parallel reaction from the *y cv v f* parent was also sequenced with the same primers. A single mutation was found in each allele.

Genomic rescue constructs and germline transformation: A Drosophila genomic library in λEMBL3 (provided by Iain Dawson, Yale University) was screened with an *sl* cDNA probe. A positively hybridizing clone (λG1) was purified by two rounds of rescreening, genomic DNA was prepared, and a restriction map of the λG1 insert was generated (Sambrook *et al.* 1989). An ~10-kb *Sal*I/*Xba*I fragment (X10) was identified within λG1, which includes the entire *sl* transcription unit and no others, by comparing the restriction map to a published map (Jones and Rubin 1990) and to the genomic sequence of the region (Adams *et al.* 2000). The X10 fragment was subcloned into pBluescript KS, reisolated by a *Kpn*I/*Not*I digest (enzymes that do not cut within X10, but which cleave on either side of the *Xho*I and *Xba*I sites in the pBluescript polylinker), and ligated into the *Kpn*I and *Not*I sites of *pCaSpeR-4*. Modified versions of X10 were produced by site-directed mutagenesis using the Quikchange kit (Stratagene, La Jolla, CA), following the protocol supplied by the manufacturer. Mutagenic primers (5′ GATTAGCCGAGTGTATGTGAAGGGTCAACGCCT 3′ and 5′ AGGCGTTGACCCTTCACATACACTCGGCTAATC 3′ for the P1035V mutation; 5′ GATTAGCCGAGTGTATGCGAAG GGTCAACGCCT 3′ and 5′ AGGCGTTGACCCTTCGCATA CACTCGGCTAATC 3′ for P1035A) were purchased from IDT (Coralville, IA) and full-length oligonucleotides were purified by PAGE as described (Sambrook *et al.* 1989). The mutations were generated in a 1.7-kb *Bam*HI fragment subcloned from X10 and confirmed by sequence analysis of the entire 1.7-kb fragment. A modified X10 fragment was then reconstructed in pBluescript using the mutated 1.7-kb *Bam*HI fragment and recloned into the *Kpn*I and *Not*I sites of *pCaSpeR-4*. The plasmid DNA preparation for each modified construct to be injected was sequenced just prior to injection to confirm the presence of the expected mutation. Germline transformations were performed essentially as described (Cripps and Bernstein 2000); the injection buffer had a final concentration of 1 μg/μl *pCaSpeR-4::X10* and 0.1 μg/μl Δ2-3 helper plasmid in a 0.1 mm sodium phosphate buffer pH 7.8, 5 mm KCl. Injections were carried out in w^{1118} embryos and the presence of the transposon was detected in the progeny of the injectees by the presence of the w^+ gene marker of *pCaSpeR-4*. The transposon insertion was mapped using *w;Bl/CyO* or *w;Bsb/TM6* stocks and balanced lines were established. The transgenes were crossed into a $w\ sl^l$ or $w\ sl^9$ background for phenotypic analysis.

RESULTS

Isolation of seven new *sl* alleles: We screened ~20,000 EMS-mutagenized X chromosomes for new *sl* mutations in a standard F_1 screen, crossing mutagenized sl^+ males to sl^l females. Seven lines were recovered that showed a similar, X-linked recessive, *sl*-like phenotype both in *trans*-heterozygous combination with sl^l and in a homozygous condition. The simplest interpretation is that each line represents a new *sl* allele; the seven mutations were therefore designated $sl^4 \ldots sl^{10}$.

***sl*^{7} and *sl*^{9} eye phenotypes differ from previously isolated alleles:** Adult eyes from females homozygous for all seven new alleles were sectioned and stained with toluidine blue to reveal photoreceptor cell fate. We found that five of the seven new alleles ($sl^{4,5,6,8,10}$) showed a similar percentage —ranging between ~30 and 50%—of ommatidia with extra-R7 cells, as we previously described (Thackeray *et al.* 1998) for the older alleles (Figure 1, A–E, and Figure 2A). However, one allele, sl^9, showed a slightly higher frequency, with a mean of 58.9% of mutant ommatidia. Indeed, of the six sl^9 eyes in our sample, three had >80% of ommatidia with extra-R7 cells; by contrast, only one other eye of any other genotype even showed 65% of mutant ommatidia. Although the small size of the samples prevents a meaningful statistical comparison, these results and those described below suggest that the sl^9 allele has a more extreme eye phenotype than that of any other previously characterized *sl* allele. The remaining allele, sl^7, showed a very low level of mutant ommatidia compared to that of all the others, with a mean of only 7.1% showing one or more extra-R7 cells.

The wing length of females homozygous for all seven new alleles was comparable to the length previously de-

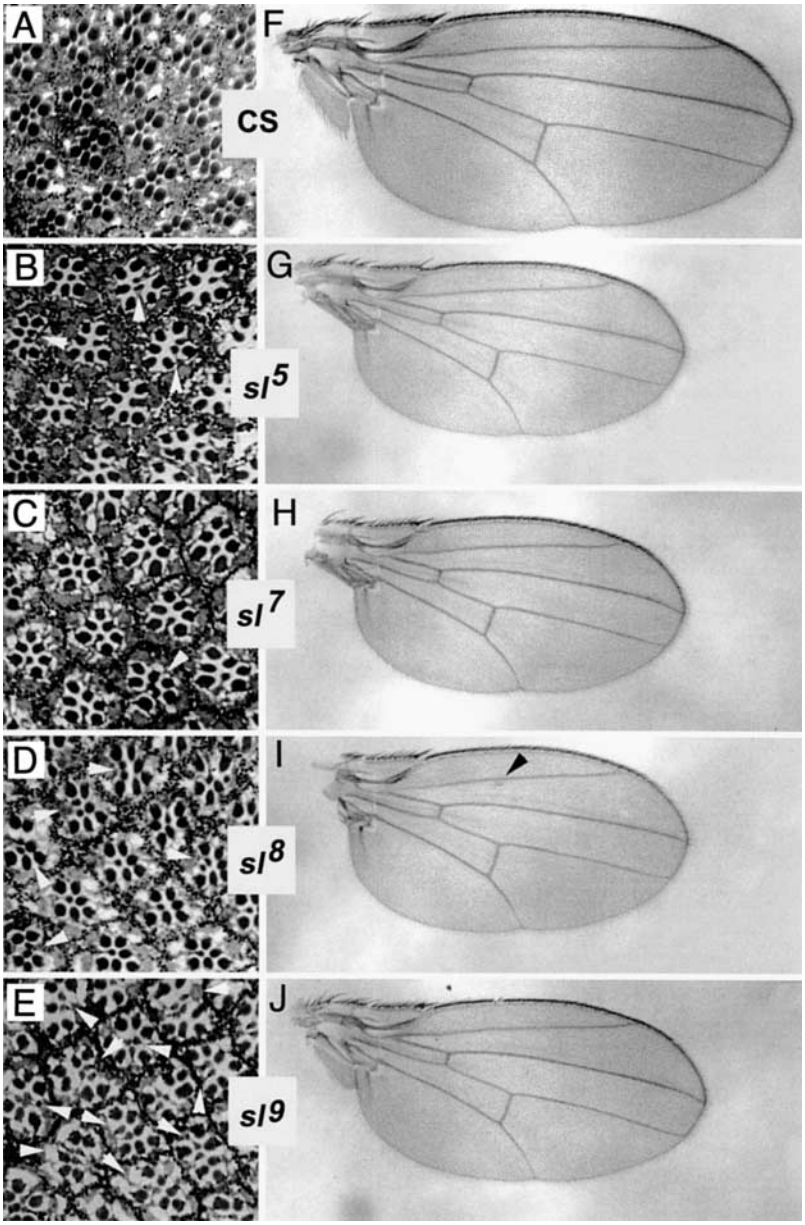


Figure 1.—Eye and wing phenotypes in the new *sl* alleles. Plastic sections of adult eyes stained with toluidine blue (left column) and wings (right column) are shown from females of the Canton-S strain used for outcrossing (A and F) and from females homozygous for sl^5 (B and G), sl^7 (C and H), sl^8 (D and I), and sl^9 (E and J). Open arrowheads indicate a mutant ommatidium containing one or more extra-R7 cells. Note in E the very high frequency of mutant ommatidia in the sl^9 homozygote. The solid arrowhead in I indicates an example of ectopic wing vein that appears in some wings from homozygous animals of all seven of the new alleles.

scribed for sl^1 and sl^2 (Thackeray *et al.* 1998) at ~80% of the normal length (Figure 1, F–J, and Figure 2B). Adults homozygous for all three of the preexisting *sl* alleles contain ectopic wing veins, principally beside vein LII, as well as less frequently in the third posterior cell or near the posterior crossvein (Thackeray *et al.* 1998). Ectopic veins were observed in wings from females homozygous for each of the seven new alleles at comparable or slightly lower frequency (10–40% of wings) and in positions identical to the original alleles. The frequency and extent of ectopic wing-vein formation is rather variable even within a given *sl* genotype (R. Mankidy and J. R. Thackeray, unpublished data), so we did not attempt to quantify this phenotype more precisely in the new alleles.

The sl^7 phenotype shows that the eye and wing pathways are separable: Because the *sl* phenotype can be partially suppressed by uncharacterized modifiers (R. Mankidy and J. R. Thackeray, unpublished observations), we outcrossed a stock carrying the sl^7 mutation again to determine whether the very mild eye phenotype of this allele is genuine; as a control, we outcrossed a stock carrying the sl^6 mutation in a parallel set of crosses. After two further rounds of outcrossing and reisolation of the chromosomes carrying the sl^6 and sl^7 mutations, the eye and wing phenotypes were determined again. In each case the results were consistent with the original analysis: wing length was in the typical range seen for *sl* homozygotes (sl^6 mean = 1.07 mm; sl^7 mean = 1.24 mm, n = 30 for both genotypes), while 43.1% of sl^6 (n = 4, SEM = 4.3) and 3.7% of sl^7 (n = 6, SEM = 1.7) ommatidia from homozygous females had one or more extra-R7 cells. This confirms that the unique combination of a

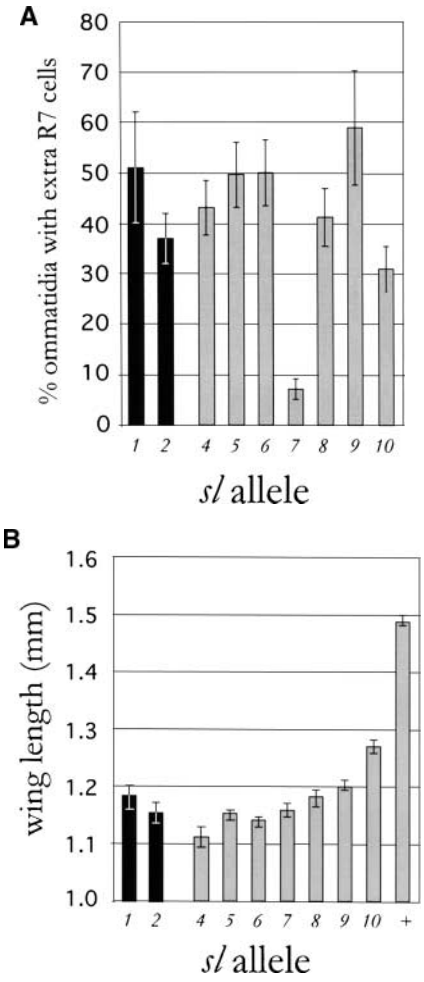


FIGURE 2.—Histograms of eye and wing defects in the new *sl* alleles. (A) Percentage of ommatidia containing one or more extra-R7 cells in homozygous females of each genotype; $n = 6$ for each genotype, and error bars show the standard error of the mean. The solid bars indicate data described elsewhere (THACKERAY *et al.* 1998). The numbers on the *x*-axis refer to the allele designation. (B) Wing lengths of homozygous adults; $n = 30$, and error bars indicate the standard error of the mean.

null-length wing but almost wild-type eye is the true sl^7 homozygous phenotype and shows that the eye and wing phenotypes are genetically separable.

To characterize the nature of the sl^7 allele in greater detail, we examined it in *trans*-heterozygous combination with two other alleles, sl^8 and sl^9. *Trans*-heterozygous adults in all three combinations—sl^7/sl^8, sl^7/sl^9, and sl^8/sl^9—had wing lengths indistinguishable from each other and typical of homozygotes for any single allele (Figure 3, E–H). In the eye, however, the sl^8/sl^9 *trans*-heterozygotes showed a phenotype equivalent to sl^8 homozygotes: 45.9% of ommatidia had extra-R7 cells (SEM = 9.0, $n = 6$), whereas sl^7/sl^8 and sl^7/sl^9 *trans*-heterozygotes both showed a phenotype indistinguishable from sl^7 homozygotes: 10.2% (SEM = 1.7, $n = 6$) and 7.8% (SEM = 2.6, $n = 5$) of ommatidia had extra-R7 cells, respectively (Figure 3, A–D and I). Because the severity of the eye phenotype of sl^7/sl^8 and sl^7/sl^9 females is not simply intermediate between the values seen in the homozygotes (Figure 2A), these results suggest that the $sl^{7,8,9}$ alleles do not differ simply by their quantitative level of Sl activity; instead, the sl^7 allele appears to provide a PLC-γ function that is missing from both sl^8 and sl^9 during photoreceptor cell development. Furthermore, because sl^7 is clearly equivalent to sl^8 and sl^9 with respect to the developmental pathway(s) affecting the length of the wing blade, these results imply that the role of Sl during development differs in some way in the ommatidia and the wing.

The *sl* mutations are scattered throughout the ORF: We scanned the *sl* transcription unit between the start and stop codons in each of the new alleles by SSCP, looking for changes relative to the parental X chromosome. A single change was found in each allele, as illustrated in Figure 4A. Four of the seven alleles, sl^4, sl^6, sl^9, and sl^{10}, contain a lesion expected to produce a truncated Sl product. Among this group sl^9 is particularly revealing, because the stop codon in this allele occurs in codon number 54 out of 1236. Any mutant Sl protein produced in sl^9 homozygotes would therefore lack all recognized PLC-γ domains, clearly indicating that sl^9 is a null allele. The mutation in the sl^5 allele is an in-frame deletion of 15 bp that removes a five-amino-acid segment from the C2 domain, indicating that this domain is indispensible for Sl function. The two remaining alleles both contain missense mutations in a catalytic domain: sl^7 has a P1035L mutation within region Y and sl^8 has a G385D mutation within region X.

Further mutagenesis of Pro^{1035}: Because the sl^7 mutation may identify a region that is differentially involved in eye and wing PLC-γ signaling, we investigated this mutation further. Position 1035 in Sl is homologous to a site within region Y that contains a proline in all PLC subtypes (β, γ, δ, and ε) described to date from plants, yeast, Drosophila, and mammals (Figure 4B; ELLIS *et al.* 1998). Analysis of the crystal structure of rat PLC-δ1, the only PLC for which a structure has been determined, places the homologous proline (Pro^{552}) immediately adjacent to one of three loops that form a hydrophobic ridge surrounding the active site. The ridge is thought to insert into the membrane, allowing phospholipid substrates access to the active site of the enzyme (ESSEN *et al.* 1996). Because the X and Y catalytic domains are highly conserved in all PLC proteins and are therefore likely to be structurally and functionally similar in all four PLC subtypes, this proline probably serves the same structural role in Sl. The sl^7 mutation might therefore affect the ridge in at least two ways: loss of the proline might twist part of the ridge into a less favorable orientation or the increased hydrophobicity due to the introduction of a leucine could alter its interaction with the membrane or substrate in some way.

To determine whether the sl^7 phenotype is generated

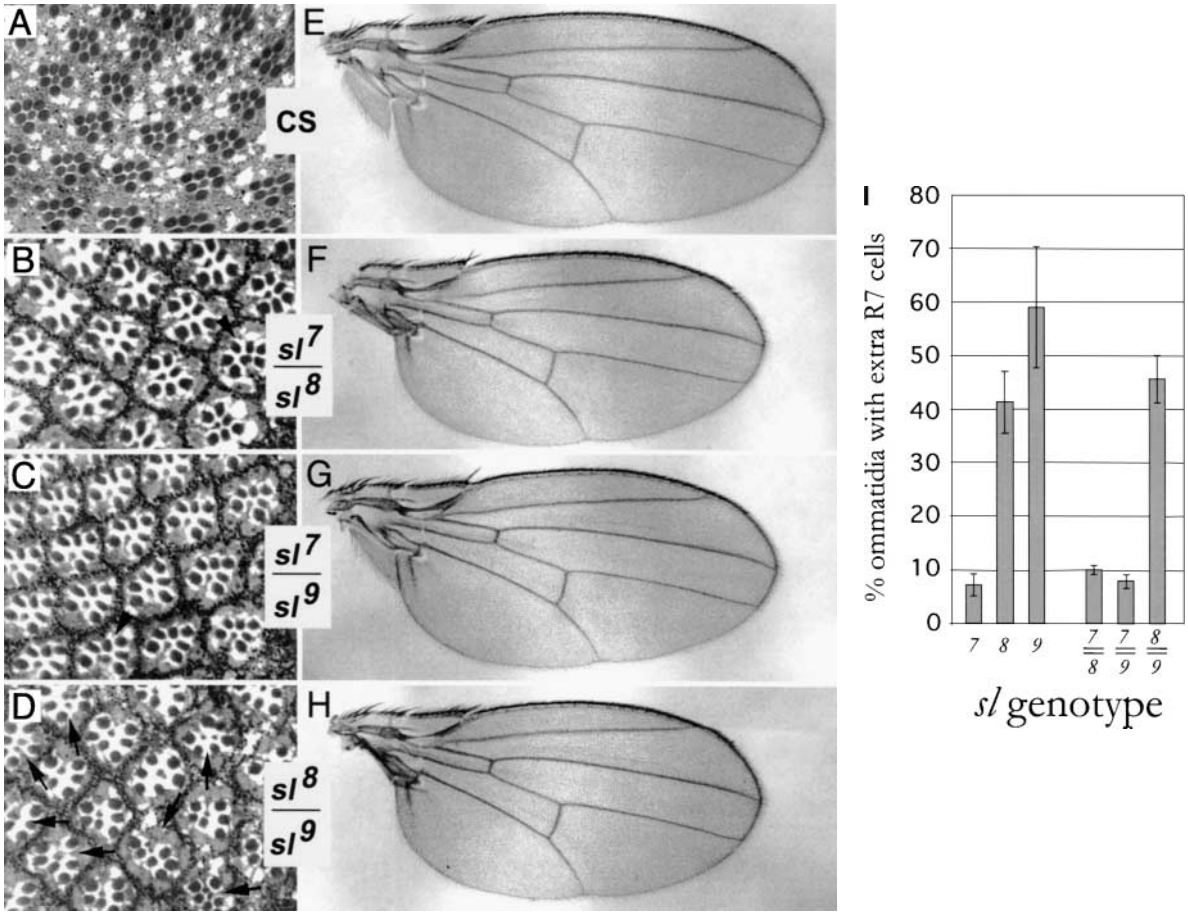
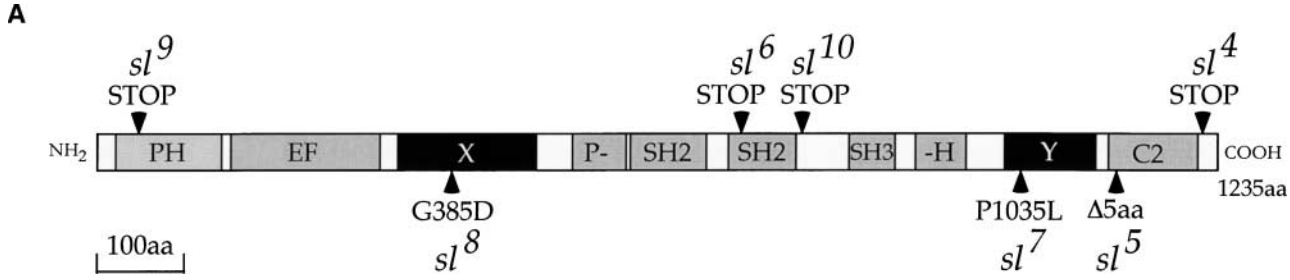


FIGURE 3.—Eye and wing phenotypes of *sl trans*-heterozygotes. Plastic sections of adult eyes stained with toluidine blue (left column) and wings (right column) are shown of females from the following genotypes: Canton-S (A and E), sl^7/sl^8 (B and F), sl^7/sl^9 (C and G), and sl^8/sl^9 (D and H). The arrowheads in B and C indicate relatively rare ommatidia containing an extra-R7 cell when sl^7 is *trans*-heterozygous with one of the null alleles. (I) Histogram showing the frequency of photoreceptor R7 defects in *sl trans*-heterozygotes. Error bars indicate standard error of the mean; $n = 6$ for sl^7/sl^8, $n = 5$ for sl^7/sl^9, and $n = 6$ for sl^8/sl^9. Data for homozygotes are taken from Figure 2. Note the much greater frequency of extra-R7 cells in the sl^8/sl^9 *trans*-heterozygote, indicated in D by arrows.

by the loss of the proline at 1035 or by the presence of the leucine at the same site, we followed an *in vitro* mutagenesis strategy to reveal the effect of two alternative changes to Pro^{1035}. First, we generated a germline rescue construct (X10) containing ~10 kb of genomic DNA that includes the *sl* open reading frame and several kilobases of sequence upstream and downstream of the translation start and stop codons, respectively. A single copy of X10 rescued the extra-R7 phenotype of sl^l and sl^9 homozygotes completely; for example, among 10 sl^9 homozygotes containing X10 (five individuals each from two independent insertions), only one ommatidium containing an extra-R7 cell was observed (Figure 5, A and B, and Figure 6A). The wing length of the X10-containing lines was also rescued (Figure 5, E and F, and Figure 6B), but because the size of adult tissues is heavily influenced by environmental conditions, it is intrinsically difficult to be certain whether or not the rescue is complete. However, we have previously found that the normal range for a wild-type wing is between ~1.35 and 1.5 mm when measured along vein LIII from the anterior crossvein to the wing margin (THACKERAY *et al.* 1998). The mean wing length of the two lines we examined containing a single copy of X10, in either an sl^l or an sl^9 background, was 1.49 and 1.34 mm, respectively (Figure 6B), suggesting complete rescue. These results suggest that the X10 fragment contains the complete *sl* transcription unit and all sequences necessary for its normal expression.

Next we made two alterations to the X10 construct, replacing Pro^{1035} with either valine (X10-P1035V) or alanine (X10-P1035A), and introduced each construct into *sl* mutant backgrounds. The X10-P1035V construct rescued the extra-R7 phenotype completely, with only four ommatidia among 20 heads containing an extra-R7 cell (Figure 5D and Figure 6A). By contrast, the X10-P1035A transgene showed a phenotype similar to sl^7 in the eye with a mean of 9.3% of ommatidia with one or more



B

552

δ1 human	HNVGHLSRIYPAGWRTDSSNY
δ4 rat	HNARQLCRVYPSGLRTDSSNY
δ *S. cerevisiae*	HNRRYLMRVYPHVLRYKSSNP
δ *D. discoideum*	ASQNHLLRVYPRGTRFDSSNF
β1 bovine	YNKMQLSRIYPKGTRVDSSNY
β2 human	YNKRQMSRIYPKGTRMDSSNY
norpA *D. mel.*	YNKHQLSRVYPAGTRFDSSNY
ε1 human	HTACQLLRTYPAATRIDSSNP
ε *C. elegans*	YTRDHLIRTYPSAKHYDSSNF
γ1 human	YNRLQLSRIYPKGQRLDSSNY
γ2 human	YNQKGLTRVYPKGQRVDSSNY
Sl *D. mel*	YHRNQISRVYPKGQRLDSSNF

1035

FIGURE 4.—The location and nature of molecular lesions in the new *sl* alleles. (A) The position of each mutation is indicated by an arrowhead either above or below a schematic representation of the Sl protein. The position of all recognized domains within PLC-γ homologs is indicated by shaded boxes. PH, pleckstrin homology domain (note that the central PH domain is split, which is indicated by the boxes containing "P-" and "-H"); EF, EF hands; X/Y, phospholipase catalytic domains; SH2, src homology 2 domains; SH3, src homology 3 domain; C2, C2 domain. The amino acid replacements generated by the sl^7 and sl^8 mutations are indicated by P1035L and G358D, respectively. (B) Alignment of Pro^{1035} in Sl with the homologous part of region Y in 11 other PLC proteins. This was produced by augmenting a previously published alignment (ELLIS *et al.* 1998) with two recently identified PLC-ε homologs and Sl. Note that the proline homologous to Pro^{1035} of Sl is conserved across all species in all PLC homologs described to date.

extra-R7 cells ($n = 10$, SEM = 1.1) in two independent lines in an sl^9 background and 2.9% ($n = 10$, SEM = 0.7) with the same two insertions in an sl^1 background (Figure 5C and Figure 6A). Wings from lines carrying both the X10-P1035V (Figure 5H and Figure 6B) and the X10-P1035A contructs (Figure 5G and Figure 6B) were at the lower end of the range typical of wild-type animals, suggesting complete rescue of the wing phenotype in each case. These data show that the sl^7 phenotype cannot be attributed solely to the loss of proline *or* to the gain in hydrophobicity resulting from the P1035L mutation, because the P1035V construct in which both changes also occur is able to rescue *sl* null strains completely. The pivotal nature of Pro^{1035} is underscored by these results, because the P1035L (*i.e.*, sl^7), P1035A, and P1035V mutations each generate a different combination of phenotypes: P1035L gives a weak phenotype in the eye and is null in the wing, P1035A is weakly mutant in the eye but wild type in the wing, and P1035V is wild type in both tissues.

DISCUSSION

One of the major goals of this study was to determine whether the previously characterized *sl* alleles indeed represent the loss-of-function phenotype, because the three previously characterized alleles could produce a truncated protein with several domains intact. Five of the seven new alleles showed a homozygous phenotype indistinguishable from that previously described for sl^1, sl^2, or sl^3 homozygotes. We found that the molecular defects in the new alleles vary widely in location within the protein, occurring at both N and C termini with different degrees of truncation and including an in-frame deletion and a single amino acid substitution among them. The most telling allele of all in this regard is sl^9, which contains a nonsense codon at amino acid 54. Because mRNAs containing premature stop codons tend to be degraded by the nonsense-mediated decay pathway (HENTZE and KULOZIK 1999), it is unlikely that many sl^9 transcripts would survive to be translated; however, even if some sl^9 mRNAs escaped degradation, Sl products translated from them would have no recognized domains remaining. Therefore, the sl^9 mutation clearly represents a true null allele. The sl^9 homozygous phenotype is qualitatively the same as the previously characterized *sl* alleles (*i.e.*, with extra-R7 cells and ectopic wing veins), which confirms our previous results showing that Sl is a negative regulator of the RTK pathways involved in cell-fate decisions during ommatidial and wing-vein development.

The phenotype of sl^9 homozygotes is identical to that of $sl^{1,2,3,4,5,6,8,10}$, except that it is slightly more extreme in the eye. Why sl^9 homozygotes should show a more extreme phenotype than that of the other alleles is uncertain; one possibility that we cannot rule out is that a closely linked enhancer of the extra-R7 phenotype is present in the sl^9 background. However, this allele is the only one unlikely to produce any protein; every other allele could produce either a mutated full-length protein or a truncated protein containing intact copies of the N-terminal PH domain, EF hand region, region X, and the N-terminal SH2 domain. Because mammalian PLC-γ does not depend on its lipase function for its role in mitogenesis (SMITH *et al.* 1994; P. S. HUANG *et al.* 1995; YE *et al.* 2002), truncated Sl proteins lacking

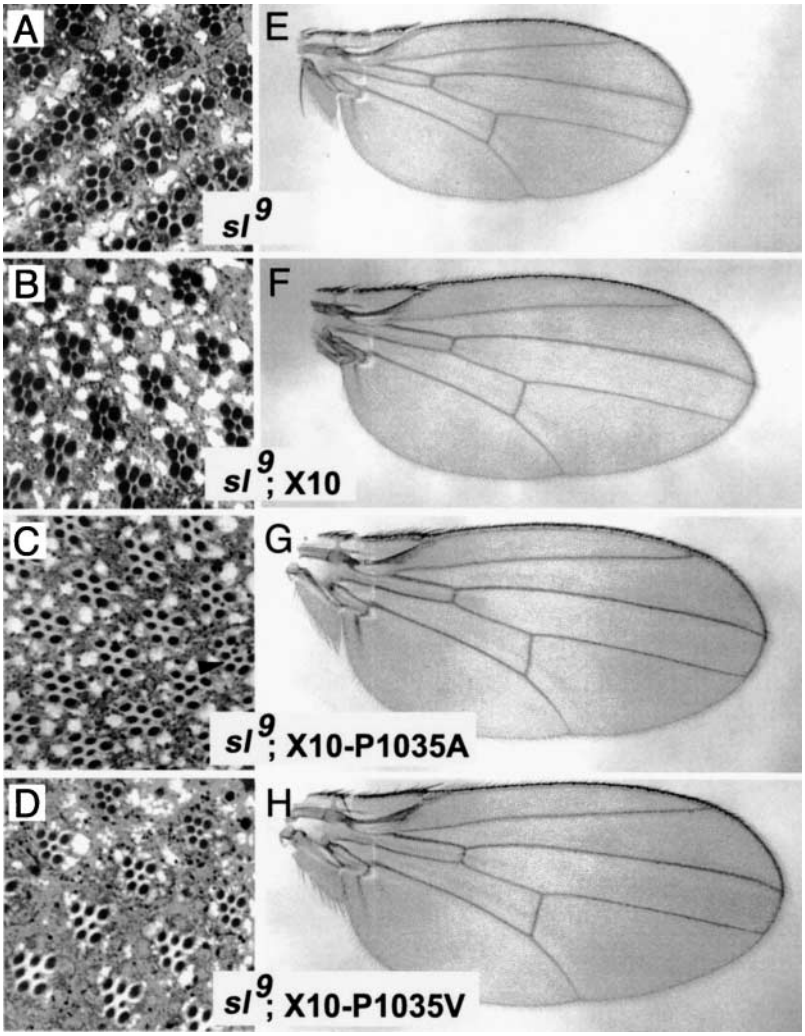


FIGURE 5.—Eye and wing phenotypes of sl^9 homozygotes expressing various *sl* genomic constructs. Plastic sections of adult eyes stained with toluidine blue (left column) and wings (right column) are shown from females of the following genotypes: sl^9 (A and E) and an sl^9 sibling expressing a wild-type *sl* genomic construct, X10 (B and F); sl^9 expressing a P1035A version of X10 (C and G); and sl^9 expressing a P1035V version of X10 (D and H). The arrowhead in C shows an ommatidium containing an extra-R7 cell due to incomplete rescue by the P1035A mutation; note the complete rescue evident in the eye section from the line expressing the P1035V construct.

intact X and Y catalytic domains (such as those that could be generated by the alleles other than sl^9) might still be able to participate at some level in certain aspects of PLC-γ function. For example, the intact N-terminal SH2 domain in each of the truncated Sl proteins would likely still be able to bind to an activated RTK at the membrane, where either its N-terminal PH domain or its EF hands might have an effect on signaling, perhaps by binding phospholipids or Ca^{2+}.

Three of the new alleles, sl^5, sl^7, and sl^8, contain either a missense mutation or an in-frame deletion, identifying functionally important residues. In the case of sl^5, a five-amino-acid deletion at the N-terminal end of the C2 domain removes five conserved residues within β-strand 2/1 of the eight β-strand C2 structure (RIZO and SUDHOF 1998). C2 motifs typically bind calcium and phospholipids, but have proven to be capable of interacting with a wide range of other molecules, sometimes independently of calcium binding (RIZO and SUDHOF 1998). The C2 domains of PLC-γ proteins appear to lack several residues shown to be necessary for calcium binding in the PLC-δ1 C2 domain (ESSEN *et al.* 1996; REBECCHI and PENTYALA 2000), suggesting that the PLC-γ C2 domain has another (as yet unidentified) function. Combined with the fact that the C2 domain is well conserved in all PLC-γ proteins, including within the genus Drosophila (MANNING *et al.* 2003), the near-null phenotype observed in sl^5 homozygotes indicates that the C2 domain does retain an indispensible function in Sl. The sl^8 mutation replaces Gly^{385} with aspartate within the region X catalytic domain. There is a glycine at the homologous position in all described PLC proteins (MANNING *et al.* 2003), and the immediately adjacent histidine on the N-terminal side of this glycine is required for PIP_2 hydrolysis in both PLC-δ (CHENG *et al.* 1995) and PLC-γ (SMITH *et al.* 1994), playing a direct role in catalysis by acting as a proton donor (ESSEN *et al.* 1996). It is therefore not surprising that the introduction of an aspartate at this critical location would disable the phospholipase activity of the enzyme.

By far the most intriguing of the new alleles is sl^7, because flies homozygous for this allele show the null wing-length phenotype, but have an almost wild-type eye. A trivial explanation of this phenotype might be

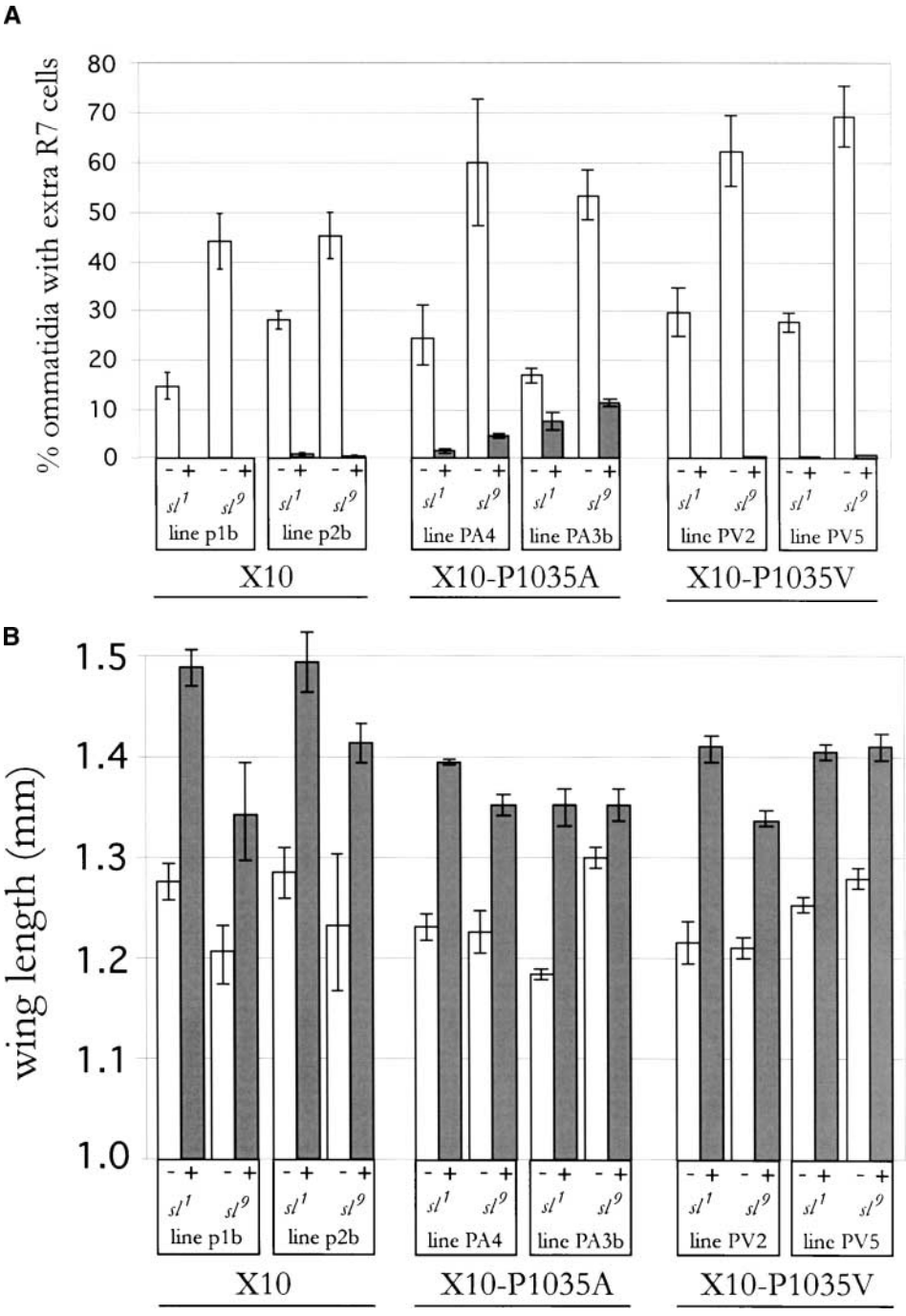


FIGURE 6.—Histograms of eye and wing defects in *sl* backgrounds expressing *sl* rescue constructs. (A) Frequency of photoreceptor R7 defects among siblings in backgrounds homozygous for either sl^{1} or sl^{9}, lacking (−) or expressing (+) the X10 *sl* genomic construct, or the X10-P1035A or X10-P1035V modified constructs. Shaded bars also indicate sibs that contained the transgene. For each background the results from analysis of two independent insertion lines are shown. Error bars indicate standard error of the mean. (B) Wing lengths of the same animals shown in A; $n = 30$ for each genotype, and error bars indicate standard error of the mean.

the presence of a closely linked modifier that suppresses the extra-R7 phenotype, but not the wing-length phenotype. However, the fact that the X10-P1035A construct produced an almost identical phenotype in the eye, in an unmutagenized background, suggests that the sl^{7} phenotype is a genuine reflection of altered PLC-γ activity rather than an artifact produced by an interacting mutation. Furthermore, there is a precedent for an *sl* allele with an sl^{7}-like phenotype: sl^{34} was isolated by Gottschewski in 1934 and was described as having short wings, but normal eyes (LINDSLEY and ZIMM 1992); unfortunately, sl^{34} has been lost. The fact that an independently isolated allele shows this same combination of eye and wing phenotypes tends to suggest that the sl^{7} mutation is not simply a rare hypomorph with threshold activity, but may in fact be a member of a class of *sl* mutations that are able to separate the extra-R7/ectopic wing-vein and wing-length phenotypes. This model is also consistent with evidence for two distinct Sl-mediated pathways in the eye and wing: a partial loss-of-function mutation of the *rl*-encoded MAPK, rl^{1}, is able to suppress both the extra-R7 and ectopic wing-vein phenotypes of *sl* ho-

mozygotes, but does not suppress the short-wing phenotype (Thackeray *et al.* 1998).

The sl^7 mutation is a replacement of Pro^{1035} by leucine within the region Y catalytic domain. We found that an alanine at the same position recapitulated the phenotype of sl^7 in the eye, but not in the wing, in which P1035A rescued the *sl* wing phenotype. By contrast, an *sl* construct containing valine at position 1035 was equivalent to one containing the wild-type proline, rescuing both the eye and wing defects of either sl^1 or sl^9. First of all, the ability of the X10-P1035V construct to rescue flies lacking *sl* function is very surprising, because this proline is one of a small number of absolutely conserved sites across all PLC isoforms in region Y. In PLC-δ1 the homologous proline is predicted to form a turn leading into one loop of a hydrophobic ridge that lines the active site, a role that might be expected to depend on a proline at this position. A possible explanation is that because valine has a side chain slightly more hydrophobic and compact than that of leucine it is drawn into the ridge by hydrophobic clustering, thereby partially overcoming the loss of proline. The slightly lower hydrophobicity and longer side chain of leucine may be unable to achieve this effect, possibly because of steric clashes due to its length. In contrast, alanine is much less hydrophobic than either valine or leucine and so may be unable to overcome the loss of the turn resulting from replacement of the proline. In any event, the fact that three different amino acids at position 1035 can produce three different combinations of eye and wing phenotypes clearly indicates that Pro^{1035} has a key modulatory role in Sl signaling.

Pro^{1035} of Sl is homologous to Pro^{552} of human PLC-δ in a region close to the active site where several mutations have been made and tested *in vitro* (Wang *et al.* 1996; Ellis *et al.* 1998). One of the most interesting of these PLC-δ mutations was a replacement of Arg^{549} by alanine; this change dramatically reduces PIP_2 hydrolysis, but has little effect on hydrolysis of PI, demonstrating that changes in this part of region Y can alter the substrate specificity of PLC-δ. All PLC enzymes are thought to be able to hydrolyze PIP_2, PIP, and PI (Ryu *et al.* 1987), although the relative physiological importance of the different substrates is unknown for any PLC. If the sl^7 mutation has an effect similar to the Arg^{549} mutation of human PLC-δ1, hydrolysis of one substrate necessary for Sl-mediated signaling in the wing might be reduced, whereas the hydrolysis of a different substrate more important for signaling during photoreceptor development is less affected. An alternative explanation is that in sl^7 homozygotes Sl is partially functional at a threshold of activity that is almost sufficient for wild-type function in the eye, but is not quite enough in the wing. However, the phenotype of sl^7/sl^8 and sl^7/sl^9 *trans*-heterozygotes suggests that there is a qualitative difference between these alleles, consistent with the sl^7-encoded PLC-γ protein lacking a function needed in the wing, but retaining a function needed in the eye. Direct assays of phospholipid hydrolysis will be required to determine whether differential substrate use by Sl occurs in different physiological contexts.

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