skittles, a Drosophila Phosphatidylinositol 4-Phosphate 5-Kinase, Is Required for Cell Viability, Germline Development and Bristle Morphology, But Not for Neurotransmitter Release

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ABSTRACT

The phosphatidylinositol pathway is implicated in the regulation of numerous cellular functions and responses to extracellular signals. An important branching point in the pathway is the phosphorylation of phosphatidylinositol 4-phosphate by the phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to generate the second messenger phosphatidylinositol 4,5-*bis*-phosphate (PIP2). PIP5K and PIP2 have been implicated in signal transduction, cytoskeletal regulation, DNA synthesis, and vesicular trafficking. We have cloned and generated mutations in a Drosophila PIP5K type I (*skittles*). Our analysis indicates that *skittles* is required for cell viability, germline development, and the proper structural development of sensory bristles. Surprisingly, we found no evidence for PIP5KI involvement in neural secretion.

PHOSPHOINOSITOL lipids have been postulated to play important roles in various cellular processes including growth, differentiation, and vesicular secretion. The phosphatidylinositol pathway consists of a series of conversions of phosphatidylinositol into singly, doubly, and triply phosphorylated products (reviewed in Carpenter and Cantley 1990, 1996; Divecha and Irvine 1995). An important branching point in the pathway occurs when phosphatidylinositol 4-phosphate (PtdIns[4]P) is phosphorylated to become phosphatidylinositol 4,5-*bis*-phosphate (PtdIns[4,5]P_2 or PIP2), a step catalyzed by phosphatidylinositol 4-phosphate 5-kinase (PIP5K; Boronenkov and Anderson 1995; Ishihara *et al.* 1996). There are two types of PIP5Ks (PIP5KI and PIP5KII) with distinct biochemical and immunohistochemical properties, but they both catalyze the conversion of PtdIns[4]P into PIP2 (reviewed in Loijens *et al.* 1996). The hydrolysis of PIP2 by phospholipase C (PLC) produces the second messengers diacylglycerol (DAG) and inositol *tris*-phosphate (IP3). DAG is an activator of protein kinase C (PKC), and IP3 plays an important role in the release of intracellular calcium (Rana and Hokin 1990). In addition, PIP2 is converted into phosphatidylinositol 3,4,5-*tris*-phosphate, which activates some PKC isoforms (Toker *et al.* 1994).

PIP2 is itself a second messenger that has been implicated in the modulation of the function of cytoskeletal regulatory proteins such as profilin, cofilin, fascin, and gelsolin (Janmey 1994). There is also evidence that phosphoinositide metabolism is involved in signal transduction and cytoskeleton regulation via the interaction with the Rho family of small G proteins (Chong *et al.* 1994; Ren *et al.* 1996). Other work has suggested an interaction between phosphoinositides and receptor tyrosine kinases (Cochet *et al.* 1991). It has also been suggested that PIP5K function may be associated with, or required for, DNA synthesis and cell proliferation (Divecha *et al.* 1993). Finally, PIP5KI was shown to be required for vesicular secretion in PC12 cells (Hay *et al.* 1995), while PIP5KII appears to be involved in vesicular trafficking in the budding yeast (Yamamoto *et al.* 1995). Most of our understanding of how PIP5Ks function to regulate cellular processes is derived from *in vitro* data. Whether the various, and apparently distinct, functions in which PIP5K is thought to be involved are related remains unknown. It also remains to be established whether these postulated roles of PIP2 are relevant *in vivo* and how the modulation of PIP5K levels affects development in animals.

The recent identification of a PIP5KI [*skittles* (*sktl*)] in Drosophila (Knirr *et al.* 1997a) makes the genetic and developmental analysis of the *in vivo* requirements of this gene possible and allows us to understand the role(s) played by phosphoinositides in various tissues and cell types. Our data show that *sktl* is essential for cell and organism viability and that it is required for cytoskeletal regulation during sensory structure development. We also find that *sktl* is required for germline development.

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Finally our analysis resolves an issue pertinent to the function of another gene, *inscuteable* (*insc*). *sktl* maps to the first intron of *insc*, whose function is required for cell fate determination during neuronal and myogenic lineage development (Kraut *et al.* 1996; Knirr *et al.* 1997b; Ruiz-Gomez and Bate 1997; Carmena *et al.* 1998). To date, all studies on neuronal *insc* function have been carried out using deletion alleles that remove or affect both genes, allowing for the possibility that the described phenotypes may be in part due to the loss of *sktl* or from the combined loss of *sktl* and *insc*. In this study we show that the loss of *sktl* is not responsible for the *insc* phenotype.

MATERIALS AND METHODS

Molecular biology: cDNA isolation, sequencing, and Northern analysis were done as described in Sambrook *et al.* (1989).

Genetics: Mutations in *sktl* were generated by the imprecise excision of a revertible *P*-element insertion in the *fata morgana* (*fam*) complementation group that affects peripheral nervous system development (Kania *et al.* 1995; note that except where a genotype is given, the *P*-element insertion will be referred to as fam^{k07505}). fam^{k07505} was mobilized to generate imprecise excisions as follows: *yw; famP{lacZ,w+}/CyO* males were crossed to *yw; Ki,Δ2-3/TM3* females. *yw; famP{LacZ,w+}/+*; *Ki,Δ2-3/+* males were individually crossed to *yw; Bl/CyO* females. Individual white eyed *yw; Δfam/CyO* males were crossed back to *yw; Bl/CyO* females to establish stocks. Stocks lacking non-Curly flies were kept for further analysis.

Due to the presence of two genes, and several independently isolated alleles of each within the locus, some nomenclature issues must be clarified. All *skittles* alleles are denoted using the name of the gene (*sktl*). *inscuteable* alleles are referred to in this article using the gene name (*insc*); however, they are allelic to the *nem* mutations described by Knirr *et al.* (1997b). Thus *nem* and *insc* are interchangeable. *fata morgana* (*fam*) is a complementation group described by Kania *et al.* (1995) that affects both genes simultaneously and therefore cannot be used to describe either gene independently. The following mutant strains were used in this study:

sktl alleles: *yw;* $sktl^{\Delta 5}$*/CyO*, *yw;* $sktl^{\Delta 15}$*/CyO*, and *yw;* $sktl^{\Delta 20}$*/CyO* (this study)

insc alleles: $insc^{8}$*/CyO* and $insc^{22}$*/CyO* (Burchard *et al.* 1995; Knirr *et al.* 1997b)

Alleles that affect both *sktl* and *insc*: *famP{lacZ,w+}/CyO*, (Kania *et al.* 1995), a *P*-element insertion in the *sktl* locus; this study, and *P49/CyO* (Kraut and Campos-Ortega 1996), a deletion creating null alleles in both *sktl* and *insc*.

Lethal phase identification: To identify stages of larval lethality we balanced *yw; sktl* alleles with *CyO, y+*. Homozygous mutant larvae are therefore *yellow* and are identified by the color of their mouth hooks.

Clonal analysis: The cross used to generate mitotic clones in the wing disc was as follows: *yw, hsFLP; P{*ry+*neoFRT42D},P{y+,ry+44B}/CyO* X *yw/yw; P{ry+neoFRT42D},* $sktl^{\Delta 5\ or\ \Delta 5}$*/CyO*. The parental FRT stock (*yw/yw; P{*ry+*neoFRT42D},P{y+,ry+44B}/CyO*) was used to generate control flies. Flies were allowed to lay for 24 hr, removed, and the eggs aged for another 24 hr. Heat shock (37° 1 hr) was applied 48 and 53 hr after egg laying to maximize FLP activation. White non-Curly females were examined for the presence of yellow bristles.

The crosses used to generate females with a *sktl* mutant germline and clones in the eye imaginal disc were carried out as follows: *yw/yw; P{w+FRT42B},* $sktl^{\Delta 15}$*/CyO, y+* X *yw, hsFLP; P{w+FRT42B}, P{w+ovoD1}2R1, P{w+ovoD1}2R2/CyO*. Flies were allowed to lay for 24 hr and removed, and the eggs were aged for another 24 hr. Flies were heat shocked as above. Yellow, White non-Curly females produced by the above cross were examined for eye clones and mated to wild-type Canton S males or $sktl^{\Delta 15}$ males to examine the phenotypes of the progeny if any were produced. Control crosses in which the parental FRT stock was used instead of the FRT, *sktl*, were as follows: *yw, hsFLP; P{w+FRT42B}, L/CyO* X *yw/yw; P{w+}, P{w+ovoD1}2R1, P{w+ovoD1}2R2/CyO*.

Overexpression of *sktl*: To generate UAS-*sktl* flies, *yw* flies were transformed with pUAST vector (Brand and Perrimon 1993) carrying *sktl* cDNA as insert and were selected using *w+* as marker. Overexpression was carried out by crossing UAS-*sktl* to various Gal4 driver flies as described in the results section.

***In situ* hybridization:** *In situ* hybridization was carried out as described (Tautz and Pfeifle 1989). A 2.9-kb *sktl* cDNA fragment in pBluescript was used to generate antisense riboprobes.

Immunohistochemistry: The following antibodies were used as described: rabbit anti-PROSPERO (PROS; 1:1000; Vaessin *et al.* 1991), MAb22C10 (1:100; Canal and Ferrus 1986), Rabbit anti-INSCUTEABLE (INSC; 1:1000; Kraut and Campos-Ortega 1996), and Mab anti-EMBRYONIC LETHAL VISION DEFECTIVE (ELAV; 1:50; Robinow and White 1991). We made numerous attempts to raise antibodies to the SKTL protein using bacterially expressed fusion proteins and unique peptide sequences but were unsuccessful. We also tested mouse antibodies (Loijens *et al.* 1996) for cross-reactivity with Drosophila embryos without success. For 4′,6-diamidino-2-phenylindole (DAPI) staining, ovaries were fixed in 4% formaldehyde in phosphate-buffered saline and 0.1% Tween (PBT). Ovaries were washed in PBT and mounted in 75% glycerol in 100 nm Tris pH 7.5 with 1 mg/ml DAPI.

Electrophysiology at the third instar larval neuromuscular junction: $sktl^{\Delta 15}$ and fam^{k07505} flies were balanced with a translocation balancer in which the Curly (Cy) and Tubby (Tb) markers segregate together [*T(2;3)* $sktl^{\Delta 15}$*/SM5; TM6B, Tb* and *T(2;3)* fam^{k07505}*/SM5; TM6B, Tb*], thus allowing second chromosome mutations to be identified during larval stages by the absence of *Tb* ($sktl^{\Delta 15}$*/T(2:3)Cy, Tb* × fam^{k07505}*/T(2:3)Cy, Tb*). Non-Tubby third instar larvae were grown at 24°. Dissections, nerve stimulation, and recordings were performed as described (Jan and Jan 1976) in HL-3 solution (Stewart *et al.* 1994) at 2 mm Ca^{2+}. Nerves innervating the ventral body wall were cut at the ventral ganglion and stimulated using a suction electrode. Nerves were stimulated for 0.2 msec at a voltage 2.5 times greater than the threshold voltage. For neuropeptide secretion analysis (large dense core vesicle release) nerves were stimulated at 30 or 50 Hz for a train of 250 ms. Four mutant larvae were examined. Recordings were performed at muscle fiber 6 in abdominal segments 4-6. Spontaneous release [miniature excitatory junctional potential (mEJP)] was measured for ~5 min. All dissections and recordings were performed at 24°.

RESULTS

***sktl* encodes a putative PIP5KI:** *sktl* was identified as a transcription unit nested in the first (10 kb) intron of *inscuteable* (*insc*; Figure 1A; Knirr *et al.* 1997b). Both genes map to 57B on the second chromosome. The

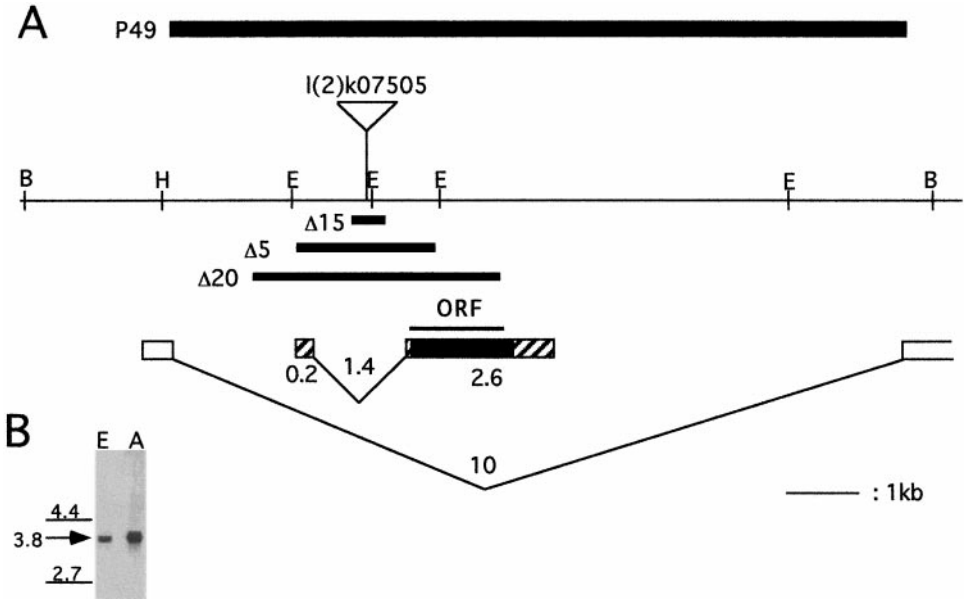

Figure 1.—Schematic representation of the *skittles* (*sktl*) locus. (A) *sktl* (▨) is located within the first intron of *inscuteable* (*insc*; □). *sktl* has two exons with a single open reading frame (■) encoding a predicted 700-amino-acid protein. A *P* element (*fam*^{7505}) is inserted in the intron of *sktl* and causes mutations in both genes. Mutations in *sktl* were created by imprecise excisions of the *fam P* element. Three excisions are shown represented by black bars. The boundaries of these excisions were not precisely mapped. Numbers indicate kilobases. *P49* is a deletion that uncovers the entire *sktl* gene and undetermined regions of *insc* (Kraut *et al.* 1996). No *sktl* RNA or INSC proteins are detectable *P49* mutants. (B) *sktl* encodes a single 3.8-kb message that is invariant throughout development (larval stages not shown). E, embryonic; A, adult.

structure and sequence of the *insc*/*sktl* genomic region, and the sizes of the *sktl* exons (Figure 1A) indicated that the protein sequence described by Knirr *et al.* (1997a) may not correspond to the full-length SKTL protein. We cloned the *sktl* cDNA by plasmid rescuing a lethal *P*-element insertion from the *fata morgana* complementation group (*fam*^{k07505}; Kania *et al.* 1995) that maps to the *sktl* locus. We used the flanking genomic fragments to screen an embryonic cDNA library (Zinn *et al.* 1989). A 2.9-kb cDNA was isolated with a single open reading frame encoding a 700-amino-acid protein (GenBank Accession number 3288870) with 59% identity to mouse and 58% identity to human PIP5KI over the entire length of the respective proteins. In contrast, the homology to human and mouse PIP5K type II isoforms was limited to 25% identity and 45% similarity. Therefore, *sktl* encodes a Drosophila homologue of vertebrate PIP5KI proteins. Interestingly, the first 100 amino acids are unique to SKTL and do not show homology to any amino-acid sequences in databases.

***sktl* has a dynamic expression pattern during development:** Northern analysis shows that *sktl* encodes a single 3.8-kb message (Figure 1B) that is invariant in size during development (data not shown). *In situ* hybridization shows that during embryogenesis *sktl* is expressed at all stages, but there is a very dynamic pattern of regulation in various developing tissues. At all stages there is a basal level of expression in all cells. At stage 5 (stages according to Campos-Ortega and Hartenstein 1985) strong expression is seen in the procephalic neuroectoderm (Figure 2A). During gastrulation, expression is elevated in the invaginating cells of the ventral and cephalic furrows (Figure 2B). At stage 11 all central nervous system (CNS) and peripheral nervous system (PNS) precursor cells express high levels of *sktl* (Figure 2C). At stage 13 most developing tissues (heart, gut, muscles, CNS, and PNS) express high levels of *skittles* (not shown). By the end of embryogenesis (stage 17) expression is prominent in a few CNS cells and the gut (Figure 2D). This expression pattern, particularly in the nervous system, is remarkably similar, if not identical, to that of *insc* (Kraut and Campos-Ortega 1996; Knirr *et al.* 1997b). This suggests that the two genes may share common regulatory elements and raises the question of whether they interact during nervous system development. Alternatively, *sktl* may be under the control of *insc* enhancers and may serve an unrelated function.

During third instar larval development *sktl* is expressed widely in all imaginal discs. In the leg disc expression is ubiquitous and uniform (data not shown). In the wing disc, expression is elevated in the precursors of the anterior wing margin sensory organs and along the anterior-posterior axis (Figure 2E). Expression is very low or absent along the dorso-ventral axis. In the eye disc, expression is elevated in the row of cells anterior to the morphogenetic furrow from which the R8 photoreceptors will differentiate (Figure 2F). In the third instar larval brain, *sktl* is expressed widely but not ubiquitously (Figure 2G). Areas of expression include the outer proliferation center of the optic lobes, several patches of cells in the midbrain, and subsets of cells in the ventral ganglion (Figure 2H).

Imprecise excisions of *fam*^{k07505} result in *sktl* mutations: The *fam* complementation group was defined by the revertible *fam*^{k07505} insertion in the *sktl* locus. Homozygous *fam*^{k07505} embryos have strongly reduced levels of

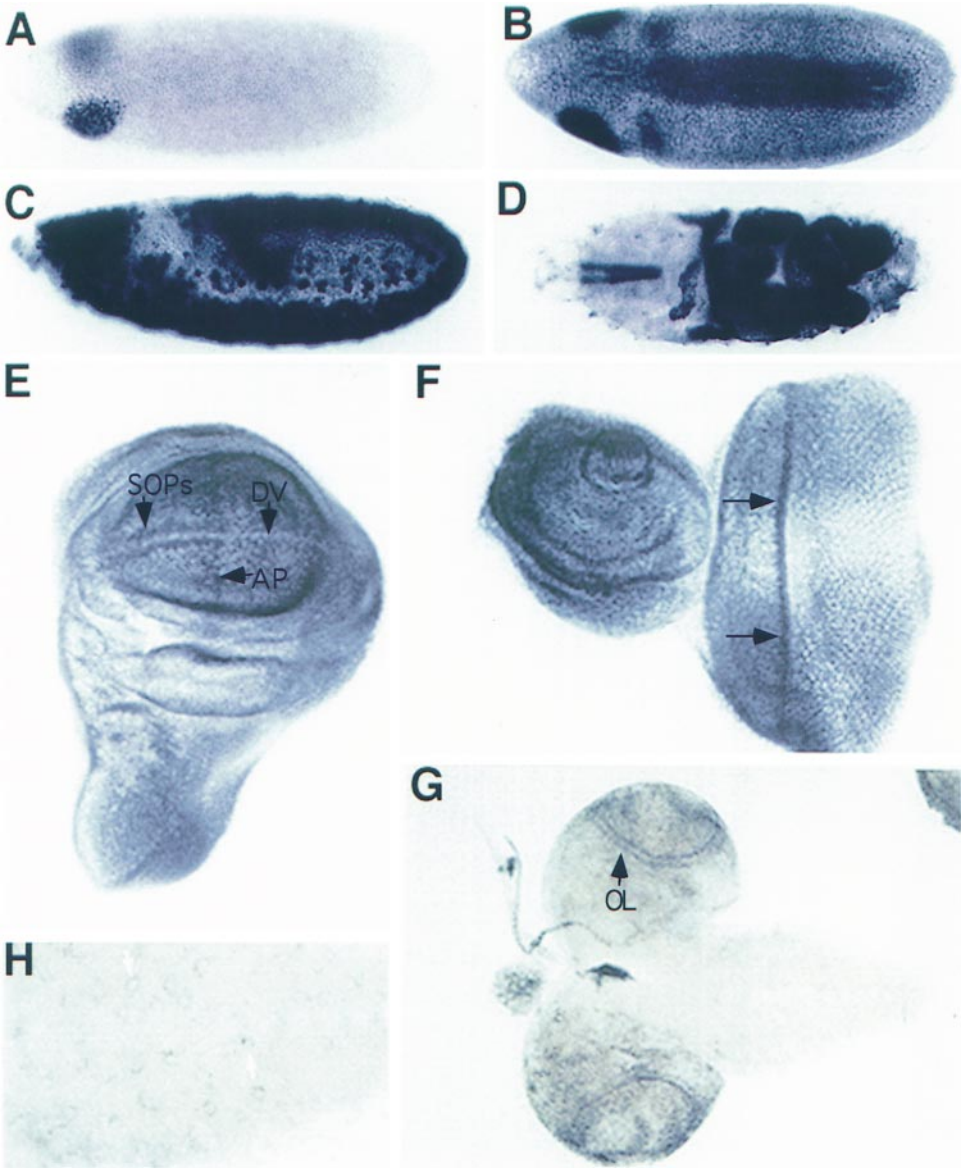

Figure 2.—*sktl* is expressed in a dynamic pattern throughout embryonic and larval development. *sktl* RNA is provided maternally (not shown). At all stages, basal levels of *sktl* are expressed in all cells. At stage 5 (A) *sktl* transcript is highly enriched in procephalic neuroblasts. During gastrulation (B) *sktl* continues to be expressed in the developing brain in addition to elevated levels of expression in the ventral and cephalic furrows. By stage 11 (C) *sktl* transcript is detectable in all neuronal precursors in the CNS and PNS as well as in migrating germ cells. Later in embryonic development *sktl* is expressed at low levels in all cells and elevated in the developing CNS, PNS, dorsal vessel, gut, and mesoderm (not shown). By stage 17 (D) *sktl* is very strongly expressed in the gut, the pharynx, and a small subset of CNS cells. In third instar larvae *sktl* is expressed in most cells of the wing (E) and eye-antennal (F) discs. In the wing disc expression is elevated in the precursors of anterior wing margin sense organs (SOPs) and along or next to the anterior-posterior axis (AP). Little expression, if any, is detectable along the dorsoventral axis (DV). In the eye-antennal disc expression is elevated in the precursors of R8 photoreceptors (arrows). In the brain *sktl* transcript is detectable in the optic lobe (OL), and several groups of cells in the midbrain (G), and many cells (arrows) in the ventral ganglion (H).

sktl transcript, suggesting that fam^{k07505} is a *sktl* allele. In addition, fam^{k07505} fails to complement two EMS alleles of *insc*, $insc^{8}$ and $insc^{22}$. Therefore fam^{k07505} represents a mutation in both *sktl* and *insc*. To create mutations that affect only *sktl* we generated imprecise excisions of fam^{k07505}. We screened for *sktl* mutations by complementation analysis with $insc^{8}$, $insc^{22}$, and fam^{k07505}. Table 1 shows the results of this screen. Nine homozygous lethal excisions were tested for complementation with $insc^{8}$, $insc^{22}$, and fam^{k07505}. Six excisions complemented $insc^{8}$ and $insc^{22}$ but failed to complement fam^{k07505} ($sktl^{\Delta 5}$, $sktl^{\Delta 9}$, $sktl^{\Delta 15}$, $sktl^{\Delta 20}$, $sktl^{\Delta 24}$, and $sktl^{\Delta 31}$). One excision ($sktl^{\Delta 13}$) failed to complement $insc^{8}$, $insc^{22}$, and fam^{k07505}. Two excisions complemented $insc^{8}$, $insc^{22}$, and fam^{k07505} ($sktl^{\Delta 25}$, $sktl^{\Delta 29}$; data not shown). Because $insc^{8}$ and $insc^{22}$ affect *insc*, and fam^{k07505} affects both *sktl* and *insc*, it follows that excisions that complement the $insc^{8}$ and $insc^{22}$ but fail to complement the fam^{k07505} are potentially mutations in *sktl* alone. Therefore $sktl^{\Delta 5}$, $sktl^{\Delta 9}$, $sktl^{\Delta 15}$, $sktl^{\Delta 20}$, $sktl^{\Delta 24}$, and $sktl^{\Delta 31}$ are good candidates for mutations that specifically affect *sktl*. To verify that these alleles do not affect *insc* we examined the expression of the *insc* RNA and protein in these mutants. We observed no detectable difference between mutant and control embryos (data not shown). Next we determined the molecular lesions associated with the excision of the *P* element in each of these alleles (Table 1). All alleles are deletions ranging between 0.7 and 4 kb. None of these deletions affects *insc* exons, and the normal pattern of *insc* expression in these alleles suggests that they do not affect any putative intronic *insc* enhancers. In contrast, all alleles showed a marked decrease in *sktl* expression (Figure 3). Therefore, imprecise excisions of the fam^{k07505} *P* element result in mutations that affect *sktl* but not *insc*, allowing us to study the function(s) of the two genes independently.

TABLE 1

***sktl* complementation and lethality**

	Δ5	Δ9	Δ13	Δ15	Δ20	Δ24	Δ31
				Complementation			
fam^{k07505}	−	−	−	−	−	−	−
insc^{8}	+	+	−	+	+	+	+
insc^{22}	+	+	−	+	+	+	+
				Deletion size			
	2.5	1	ND	0.7	4	2	1
				Stage of lethality			
Lethal	Early	Early	ND	Late	E	Early	Early
Stage	L1	L1		L1		L1	L1

Complementation of *sktl* alleles with *insc* alleles and the original *P*-element insertion used to generate the *sktl* excision alleles. All crosses were at room temperature, and stage of lethality was determined by out-crossing to wild-type *CS* background. Sizes of deletions associated with the imprecise excisions of the *P*-element insertion were determined by PCR and genomic Southern analysis. ND, not determined.

***sktl* mutations result in embryonic lethality:** To determine the function of *sktl* during embryonic development, we examined the phenotypes associated with the loss of *sktl* function using the smallest (*sktl*$^{\Delta 15}$), an intermediate (*sktl*$^{\Delta 5}$), and the largest (*sktl*$^{\Delta 20}$) excisions. *sktl*$^{\Delta 15}$ larvae are late first larval instar lethal and have no gross morphological abnormalities. Similarly, *sktl*$^{\Delta 5}$ mutants are early first instar lethal and have no obvious defects. *sktl*$^{\Delta 20}$ mutants are late embryonic lethal and also show no gross morphological defects as revealed by light microscopy (see also next section). *sktl*$^{\Delta 15}$/*sktl*$^{\Delta 20}$ and *sktl*$^{\Delta 5}$/*sktl*$^{\Delta 20}$ transheterozygotes are early first instar lethal while *sktl*$^{\Delta 20}$/*P49* transheterozygotes are identical in stage of lethality to *sktl*$^{\Delta 20}$ homozygous embryos. While these genetic data suggest that *sktl*$^{\Delta 5}$ and *sktl*$^{\Delta 15}$ are strong hypomorphs and *sktl*$^{\Delta 20}$ is a null allele, *in situ* hybridization shows that the *sktl* message, although severely reduced, is not completely abolished in *sktl*$^{\Delta 20}$ embryos (Figure 3). These observations can be reconciled by the fact that the deletion in *sktl*$^{\Delta 20}$ uncovers at least part of the open reading frame (ORF) (Figure 1A) and therefore may lack a functional SKTL protein. However, in the absence of a *sktl* antibody we are unable to confirm this prediction.

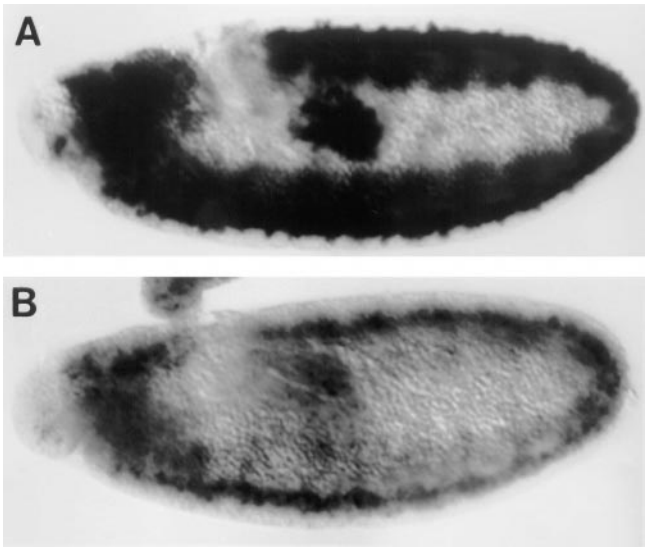

Figure 3.—*sktl* alleles have reduced levels of *sktl* RNA. Panels represent lateral views of a wild-type embryo (A) and a homozygous *sktl*$^{\Delta 20}$ embryo (B) at stage 11. Strong reduction in expression is seen in *sktl*$^{\Delta 20}$ embryos in which a large portion of *sktl* is deleted, supporting the genetic evidence that suggests that *sktl*$^{\Delta 20}$ is either a stong hypomorphic allele or a null mutation. Other alleles show degrees of message reduction consistent with the size of their respective deletions (not shown).

Zygotic *sktl* expression is not required for embryonic nervous system development: Because *sktl* is expressed at high levels in most if not all nervous system precursors, we were interested in determining the consequences of the loss of *sktl* on nervous system development. We used *sktl*$^{\Delta 20}$ mutant embryos to determine if *sktl* is required for nervous system development. Nervous system development was examined using anti-ELAV (Robinow and White 1991) and Mab 22C10 (Canal and Ferrus 1986) antibodies to detect neurons and anti-PROS to detect neuronal precursors during early neurogenesis and glial cells during late neurogenesis (Vaessin *et al.* 1991). No detectable defects were seen by stage 16 with these markers (data not shown). The absence of an *insc* phenotype in *sktl* mutants demonstrates that the phenotypes reported by Kraut and Campos-Ortega (1996) and Kraut *et al.* (1996), *i.e.*, the loss of nervous system cells due to mislocalization of Numb and Prospero during neuronal lineage development, are indeed caused by the absence of *insc* and not by the loss of function of either *sktl* alone or both *sktl* and *insc*.

***sktl* is not required for neurotransmitter release at the neuromuscular junction:** PIP5KI was shown to be required for Ca^{2+}-dependent neuropeptide secretion from PC12 cells (Hay *et al.* 1995). In addition, several

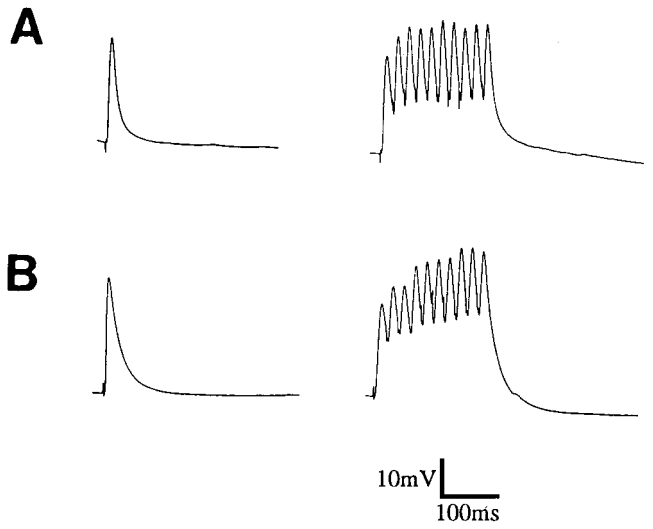

Figure 4.—Evoked excitatory junctional potentials (EJPs) in third larval body-wall muscles. EJPs were evoked by either a single stimulus (left) or a short train of stimuli at 30 Hz (right). Larval neuromuscular junction preparations were dissected as described (Jan and Jan 1976) and incubated in 1 mm $CaCl_2$ HL-3 solution (Stewart *et al.* 1994). The rapid and transient glutamatergic responses (*i.e.*, individual EJPs) are indistinguishable between control larvae (A; $sktl^{\Delta 15}/TM6$ or $fam^{k07505}/TM6$) and the mutant larvae (B; $sktl^{\Delta 15}/fam^{k07505}$). The slow ramp depolarization underlying the train of stimuli is thought to be mediated in part by the peptide PACAP (Zhong and Peña 1995). This ramp potential appears to be unaltered in $sktl^{\Delta 15}/fam^{k07505}$ larvae. The resting potential was −64 mV.

lines of evidence suggest that PIP5Ks play a crucial role in regulating membrane trafficking (reviewed in De Camilli *et al.* 1996; Martin 1997; see discussion). Furthermore, *sktl* is expressed in many cells in the ventral ganglion of third instar larvae. Some of these cells may correspond to motor neurons innervating the larval body wall muscles. The Drosophila third instar larval neuromuscular junction is an excellent system for measuring neurotransmitter release from motor neurons. To test the requirement of *sktl* for neurotransmitter release we used third instar larvae transheterozygous for the $sktl^{\Delta 15}$ and fam^{k07505} alleles. This combination results in late second instar lethality with a few third instar escapers. Electrophysiological recordings at the neuromuscular junction (Jan and Jan 1976) were done to examine spontaneous release as well as evoked release. The size, shape, and frequency of evoked and spontaneous responses were examined in control (heterozygous for either allele alone) and transheterozygous mutant larvae. Evoked response was measured after either single or repetitive stimulation (Figure 4). We also tested for nerve fatigue by repetitive stimulation. No detectable defects were observed in any of the above measurements, suggesting that *sktl* is not required for glutamate neurotransmitter release, at least at the larval neuromuscular junction. It is therefore unlikely to play a role in regulating vesicular trafficking at that junction.

Zhong and Peña (1995) showed that repetitive stimulation at 20 Hz or higher results not only in a fast evoked response but also in a slow, neuropeptide-dependent, depolarization. Neuropeptides are released by dense core vesicles from the nerve terminal. Hay *et al.* (1995) showed that PIP5KI was required for dense core vesicle secretion from PC12 cells. To test the requirement of *sktl* for dense core vesicle secretion we stimulated the motor nerves at 30 and 50 Hz. The resulting slow depolarization profile in *sktl* mutants was indistinguishable from that of heterozygous controls or wild-type larvae (Figure 4). Therefore *sktl* does not appear to play a role in regulating dense core vesicle secretion at least at the larval neuromuscular junction. It is not possible to exclude the possibility that the transheterozygous combination used in these experiments, while being strong enough to cause larval lethality, is not strong enough to have an effect on peptide release. However, this is unlikely because even viable hypomorphic mutations of numerous proteins involved in neurotransmission, such as synaptotagmin and RAS opposite (ROP), show severe electrophysiological defects (Littleton *et al.* 1994; Wu *et al.* 1998).

***sktl* is required for germline development:** Knirr *et al.* (1997a) reported that *sktl* is expressed in germ cells during oogenesis. At stage 6 (stages according to Spradling 1993), *sktl* expression is restricted to the future oocyte. By stage 9, expression is initiated in the nurse cells. At the end of oogenesis, large amounts of *sktl* transcript are present in the mature egg. To examine the function of *sktl* in germline development we generated *sktl* germline clones using one of the excision alleles ($sktl^{\Delta 15}$). Negative control crosses in which no recombination was induced resulted in 100% of the females being sterile. The ovaries of these females, carrying the dominant sterile ovo^{D1} marker, were severely atrophic and showed very early arrest of egg chamber development. Positive control crosses showed that 47% of the females were fertile (Table 2). In contrast, all *sktl* recombinant females were sterile (Table 2). Ovaries were dissected and stained with DAPI to reveal the nuclei. Oogenesis in these females was arrested after stage 10, and very few eggs were fully developed. Arrested egg chambers showed defects in nurse cell nuclei at and after stage 10 (compare Figure 5, B and C), but no defects in nuclear morphology were seen before that stage (arrows in Figure 5C). The affected nuclei appeared very small and fragmented, suggesting that *sktl* is required for nurse cell viability, and therefore proper egg chamber development. In contrast, the oocyte nucleus did not appear to be affected. The few eggs that developed were smaller than the eggs produced by control flies and showed defects in their dorsal appendages (compare Figure 5, D and E). Generally, the dorsal appendages of *sktl* mutant eggs were short and thick. The sterility associated with the partial loss of *sktl* in the

TABLE 2

***sktl* clone frequency**

	P{*y*+}		P{*w*+, *ovo*^{D1}}			
Allele	Bristle color: yellow (%)	*n*	Eye color: white (%)	*n*	Egg laying: fertile (%)	*n*
D15	23 (2)	1137	0	164	0	164
D5	7 (1)	869	ND		ND	
+	237 (76)	312	66 (45)	146	68 (47)	146

Frequency of recombination between FRT *sklt* chromosomes and FRT marker chromosomes expressed in percentage of flies showing marked clones. *n*, total number of flies scored for each genotype. Clonal analysis was done as described in materials and methods. ND, not determined.

female germline precludes the determination of the consequences of the loss of *sktl* in embryos.

***sktl* is required for cell viability and bristle differentiation:** As discussed above, *sktl* is expressed widely in the wing disc. To examine the function of *sktl* in wing disc development we generated *sktl* mutant clones using the *sktl*$^{\Delta 5}$ and *sktl*$^{\Delta 15}$ alleles (using the FLP-FRT system, see materials and methods). The absence of the yellow marker was used to identify the clones. Approximately 76% of the control flies had yellow bristle clones on their notum, legs, and wing margin (Table 2). These bristles showed no defects. In contrast, only 1–2% of the recombinant flies had yellow bristles on the notum. In addition the size of these clones was markedly reduced in comparison to control flies: each clone consisted of a single, and rarely of two bristles. The few mutant bristles recovered showed structural abnormalities ranging from a wavy shape (in most cases) to sharp bends (in a few cases; Figure 5A), suggesting cytoskeletal defects. No mutant bristles were observed on the wing margin or the legs. The very small number of clones obtained combined with the small size of each clone suggest that *sktl* is required for either cell viability, proliferation, or both during wing disc development.

To determine if loss of *sktl* is required in the eye disc, where it is abundantly expressed, we also generated *sktl* mutant clones. In control experiments, 45% of the recombinant flies had white eye clones of variable sizes. In contrast, no *sktl* mutant clones were observed (Table 2), supporting the conclusion that *sktl* function is required for cell viability or cell division in imaginal discs. It should be noted that third instar larvae transheterozygous for the *sktl*$^{\Delta 15}$ and *fam*^{k07505} alleles show no defects in the sizes of the imaginal discs and the brain (data not shown). Therefore it is more likely that the absence of *sktl* clones results from an effect on cell viability. While we favor this hypothesis, we cannot exclude the possibility that the *sktl*$^{\Delta 15}$/*fam*^{k07505} combination, while being lethal, is not severe enough to reveal a role for *sktl* in cell proliferation.

Overexpression of *sktl* affects bristle number and morphology: To further characterize the function of *sktl*, we carried out overexpression studies. We generated flies with a *UAS-sktl* construct and used it to overex-

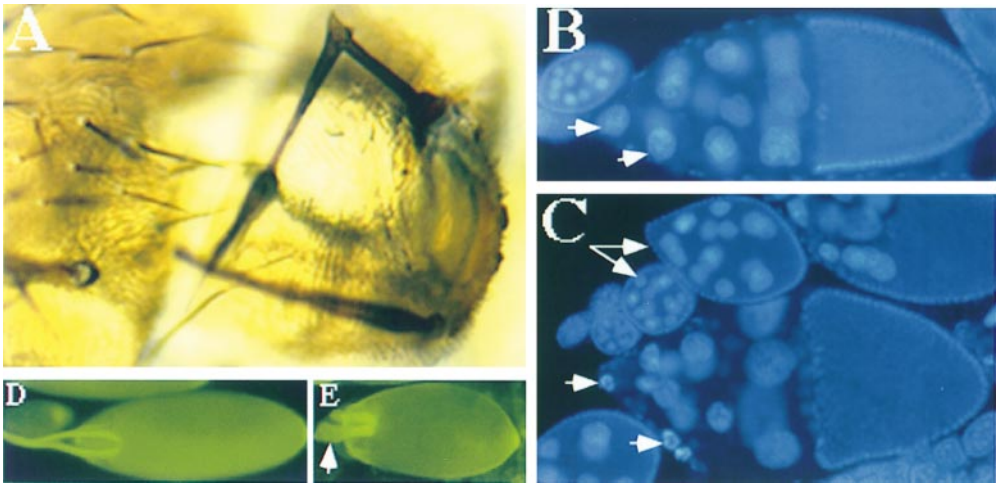

Figure 5.—Loss of function of *sktl* suggests that *sktl* is required for germline development and bristle differentiation. Clonal analysis in the wing imaginal disc results in very few, small, clones each covering one or, rarely, two bristles. These bristles have a variety of structural defects ranging from wavy shape (not shown) to sharp bends. (A) Reduction of *sktl* levels (using *sktl*$^{\Delta 15}$ allele) in the female germline results in sterile females. (B) A normal egg chamber at stage 10 of oogenesis stained with DAPI to reveal nuclei of all cells. Nurse cell nuclei (arrowheads) are normal in size and shape. In contrast, nurse cells with reduced levels of *sktl* (C) contain some small and abnormal nurse cell nuclei (arrowheads). Mutant egg chambers do not show any defects in nuclear morphology before stage 10 (arrows in C). Very few mutant egg chambers complete oogenesis. Those that do (E) are small and have short and thick dorsal appendages when compared to control eggs (D).

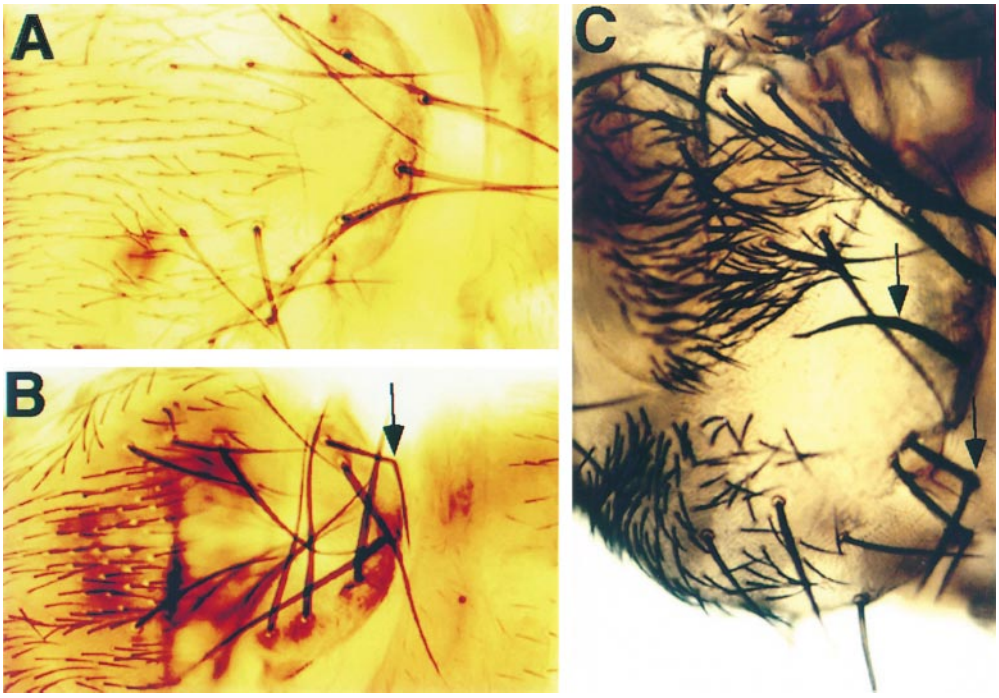

Figure 6.—Overexpression of *sktl* causes severe defects during larval development. Wild-type notum is shown in A. Overexpression in the entire wing disc (B and C) causes larval and pupal death with a few pharate adult escapers. Eclosed (B) and dissected (C) adults show ectopic microchaetae (1.7× normal number) and macrochaetae (3–6 per animal). Both normal and ectopic bristles display bends and twists (arrows), suggesting aberrant cytoskeletal architecture. Note that, contrary to wild-type flies (A), bristles on transgenic flies are in different focal planes (B), possibly reflecting problems in notum development. Some flies show severe abnormalities in the notal structure (C).

press *sktl* using a variety of Gal4 drivers (Brand *et al.* 1993). The neuronal-specific *elav*-Gal4 driver (de Simone and White 1993) resulted in no detectable phenotypes and gave rise to fertile adults. Overexpression using the ubiquitous *daughterless*-Gal4 driver (Wodraz *et al.* 1995) and the heat shock-Gal4 driver resulted in no obvious phenotypes during embryogenesis but caused early larval lethality. Expression with the ubiquitous imaginal disc driver T80-Gal4 resulted in third instar larval lethality. The lethality associated with the ubiquitous expression of *sktl*, which is itself very widely expressed, precluded the use of the UAS-*sktl* construct to rescue *sktl* mutants. Overexpression with the *dpp*-Gal4 driver (Staehling-Hampton *et al.* 1994; expressed in the morphogenetic furrow of the eye disc and along the anterior-posterior boundary in the wing disc) showed no detectable phenotypes in the eye, but caused ectopic bristle formation on the notum and wing blade. The appearance of ectopic bristles on the wing blade was seen in ~20% of the flies, but the majority of the flies (70%) showed ectopic bristles on the notum. These results prompted us to examine the effects of generalized overproduction of *sktl* in the wing disc. We used the 32B-Gal4 driver that is expressed ubiquitously at high levels in the third instar wing disc. Overexpression of *sktl* resulted in ectopic bristles (macrochaetae and microchaetae) on the notum (Figure 6B) and the wing blade (data not shown). On the notum, both the ectopic and normal bristles showed severe structural defects (Figure 6C). The structural defects of the bristles are consistent with a role for *sktl* in regulating cytoskeletal components. All ectopic bristles were associated with socket cells, suggesting that the production of ectopic bristles may be the result of the specification of extra sense organ precursors rather than the transformation of a socket cell into a bristle cell.

DISCUSSION

PIP5Ks are thought to be involved in a variety of cellular processes including cytoskeletal regulation and neuronal secretion. We generated mutations in the Drosophila PIP5K *sktl* and undertook the analysis of its function during fly development. We find that the loss of *sktl* function affects several aspects of Drosophila development and that it does not affect other aspects in which PIP5K function has been implicated.

***sktl* and vesicular trafficking:** Several studies link PIP5K activity to the regulation of vesicular trafficking (reviewed in De Camilli *et al.* 1996; Martin 1997). It is thought that PIP2 metabolism may regulate both endocytosis and exocytosis through interactions with key proteins required for both processes, namely dynamin, AP-2, AP-3, synaptotagmin, and CAPS. (Chen *et al.* 1991; Smythe *et al.* 1992; Gaidarov *et al.* 1996; Salim *et al.* 1996; Hao *et al.* 1997; Martin 1997). It has also been shown that a PIP5KI is required for Ca^{2+}-regulated dense core vesicle release from neuroendocrine cells (Hay *et al.* 1995). These data suggest that PIP2 plays a crucial role in regulation of membrane trafficking. On the basis of these observations, it is expected that mutations in a PIP5KI would result in defects in evoked neurotransmitter release. The expression of *sktl* in the larval ventral ganglion (Figure 2H) also suggested that *sktl* may play a role in neural secretion. However, we find no defects in neurotransmitter release at the third

larval instar neuromuscular junction in *sktl* mutants. While we did not find defects in peptide secretion, we cannot exclude minor defects not visible in our assays. The lack of a vesicular secretion phenotype may be due to the activity of other PIP5Ks in Drosophila. Drosophila has a PIP5K type II that maps to the tip of chromosome *4* and that appears, from preliminary expression analysis, to be expressed specifically in the late embryonic CNS (B. Hassan, unpublished results). It remains to be established whether this form of PIP5K functions in neuronal secretion. Finally, as it appears that *sktl* may function in regulating cytoskeletal processes (see below), we examined whether *sktl* mutants showed any gross morphological defects in the motor nerve terminals. No such defects were observed by light microscopy.

***sktl* and germline development:** The reduction in the level of *sktl* transcript results in female sterility, indicating that *sktl* is necessary for germline development. When examined with DAPI, *sktl* mutant egg chambers show what appear to be degenerating nurse cell nuclei. Nurse cells normally act to provide the developing oocyte with RNA and proteins necessary for various aspects of embryonic development. If *sktl* is required for the function of nurse cells, reducing its levels would be expected to result in smaller eggs, which is what is observed (Figure 5E). Interestingly, no defects were observed in the oocyte nucleus, despite the fact that *sktl* is transcribed in the oocyte even before its expression in nurse cells (Knirr *et al.* 1997a).

***sktl* and bristle development:** The strongest evidence for PIP5K requirement in the regulation of the actin cytoskeleton comes from studies in platelets. Upon thrombin receptor activation the small G-protein Rac is activated. Rac activation is thought to induce PIP5K activity that results in increased PIP2 synthesis (Hartwig *et al.* 1996). PIP2 is known to have strong binding affinities to several actin interacting proteins such as fascin, profilin, gelsolin, and α-actinin (Fukami *et al.* 1996; Hartwig *et al.* 1996; Schafer *et al.* 1996). It is thought that the interaction between these proteins and PIP2 results in the inhibition of their interaction with monomeric actin and therefore allows the nucleation of new actin filaments (Pollard 1986; Pollard and Rimm 1991). This model is probably applicable to many, if not most, cells undergoing cytoskeletal rearrangements. The Drosophila adult peripheral nervous system has cells that extend sensory bristles rich in actin, providing an excellent system for the study of the defects in cytoskeleton assembly (Tilney *et al.* 1995, 1996). Mutations in the Drosophila actin interacting proteins profilin (*chickadee*) and fascin (*singed*) show severe defects in bristle morphology (Cant *et al.* 1994; Verheyen and Cooley 1994). Loss of function of either protein results in bristles that lack actin filament integrity, causing bending and branching during extension (Verheyen and Cooley 1994; Cant and Cooley 1996). Our analysis indicates that the alteration of PIP5KI levels results in structural defects in sensory bristles, providing genetic evidence for the involvement of PIP5KIs in cytoskeletal regulation. The phenotypes observed in *sktl* clones are similar but not identical to those observed in *singed* and *chickadee* mutants (Verheyen and Cooley 1994; Cant and Cooley 1996). Specifically, while the loss- and gain-of-function of *sktl* results in bent and wavy bristles (Figure 4A; Figure 5, B and C), we did not observe branching bristles. Finally, it is interesting to note that mutations in all three genes (*chickadee*, *singed*, and *sktl*) result in female sterility. Therefore, both germline and bristle development present model systems in which to study the interactions between PIP5K and the actin cytoskeleton.

Does *sktl* have an early role in the adult PNS? The appearance of extra bristles associated with socket cells as a result of the overexpression of *sktl* in the wing disc can be explained in one of two ways: extra cell division, or specification of an extra precursor cell. The sterility resulting from the removal of the maternal component and the failure of somatic clones to survive does not allow us to correlate the ectopic production of bristles with a loss-of-function phenotype. However, it is interesting to note that cytoskeleton-associated proteins like INSC (Kraut and Campos-Ortega 1996; Kraut *et al.* 1996) and sanpodo (a Drosophila tropomodulin homologue; Dye *et al.* 1998) play a significant role in cell fate specification in the nervous system.

In conclusion, the great diversity of functions proposed for PIP5Ks and phoshatidylinositol kinases in general makes the *in vivo* analysis of their function difficult. However, the identification of these genes in a multicellular organism amenable to genetic manipulation like Drosophila (Leevers 1996; Knirr *et al.* 1997a; this study), allows us to test genetically the biochemical and cell biological data available and to uncover new roles for PIP5Ks in development. Mutations in *sktl* allowed us to test which of the many roles proposed for PIP5KI proteins are carried out by *sktl* and which may be the function of other isoforms and/or types of PIP5Ks. For instance, it appears that *sktl* is involved in regulating the cytoskeleton and possibly cell survival. In contrast, *sktl* does not appear to play a role, or plays a redundant role, in neurotransmitter or neuropeptide release, and in cell proliferation. The distinction between redundancy and lack of requirement awaits the genetic and functional characterization of other PIP5Ks in Drosophila.

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