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

Bassem A. Hassan,* Sergei N. Prokopenko,† Sebastian Breuer,† Bing Zhang,§ Achim Paululat† and Hugo J. Bellen*,†,§

*Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas, 77030, ¹Department in Developmental Biology, Baylor College of Medicine, Houston, Texas, 77030, ²Fachbereich Biologie, Philips Universitat, 35032 Marburg, Germany and ³Department of Molecular and Human Genetics, Division of Neuroscience and Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

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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-bisphosphate (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-bisphosphate (PtdIns[4,5]P2 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 triphosphate (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-trisphosphate, 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, coflin, 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 (Chung 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.

Corresponding author: Hugo J. Bellen, Howard Hughes Medical Institute, Baylor College of Medicine, Room T630, 1 Baylor Plaza, Houston, TX 77030. E-mail: hbel@bcm.tmc.edu

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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; Carmen 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 fam15755s). fam15755s was mobilized to generate imprecise excisions as follows: yw; famM(LacZ, +)/CyO males were crossed to yw; K1,l2-3/ TM 3 females. yw; famP(LacZ, +)/CyO males were crossed to yw; K1,l2-3/ TM 3 females. yw; famP(LacZ, +)/CyO males were crossed to yw; K1,l2-3/+ males were individually crossed to yw; Bl/CyO females. Individual white eyed yw; femP/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 of them, some nomenclature issues must be clarified. All sktl 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; sktl1/2/CyO, yw; sktl11/2/CyO, and yw; sktl15/2/CyO (this study)
insc alleles: insc01/2/CyO and insc21/2/CyO (Burniard et al. 1995; Knirr et al. 1997b)

Alleles that affect both sktl and insc: famP[1acZ,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{y+; ne-FRT 42D}, P{y+, ry+; 44B}/CyO X yw; yw; P{y+; ne-FRT 42D}, sktl15/2/CyO. The parental FRT stock (yw; yw; P{y+; ne-FRT 42D}, 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{y+; FRT 42B}, sktl11/2/CyO, y+ X yw, hsFLP; P{y+; FRT 42B}, P{yw+; ovd1}2R1, P{yw+; ovd1}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 sktl15/2 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{y+; FRT 42B}, L/CyO X yw; yw; P{yw+}, P{yw+; ovd1}2R1, P{yw+; ovd1}2R2/CyO.

Overexpression of sktl: To generate UAS-sktl flies, yw flies were transformed with pUAST vector (Brand and Perrimon 1995) 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 Pfleif e 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; Vaesin et al. 1991), Rabbit anti-INSCEUTABLE (INSCE; 1:100; 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: sktl11/2 and fam15755s flies were balanced with a translation balancer in which the Curly (Cy) and Tubby (Tb) markers segregate together (T;2;3) sktl11/2 SM 5; T;2;3 fam15755s/ SM 5; T;2;3 Tb, thus allowing second chromosome mutations to be identified during larval stages by the absence of T (sktl11/2T;2;3 Cy, Tb × fam15755s/ 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 23 °C. 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 PIP5KL: 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
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\textsuperscript{107505}; 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 PIP5KI 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 (Knirr 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\textsuperscript{107505} result in sktl mutations:** The fam complementation group was defined by the revertible fam\textsuperscript{107505} insertion in the sktl locus. Homozygous fam\textsuperscript{107505} embryos have strongly reduced levels of
sktl transcript, suggesting that fam^{07505} is a sktl allele. In addition, fam^{07505} fails to complement two EMS alleles of insc, insc^{6} and insc^{22}. Therefore fam^{07505} represents a mutation in both sktl and insc. To create mutations that affect only sktl we generated imprecise excisions of fam^{07505}. We screened for sktl mutations by complementation analysis with insc^{6}, insc^{22}, and fam^{07505}. Table 1 shows the results of this screen. Nine homozygous lethal excisions were tested for complementation with insc^{6}, insc^{22}, and fam^{07505}. Six excisions complemented insc^{6} and insc^{22} but failed to complement fam^{07505} (sktl^{65}, sktl^{99}, sktl^{115}, sktl^{210}, sktl^{214}, and sktl^{311}). One excision (sktl^{413}) failed to complement insc^{6}, insc^{22}, and fam^{07505}. Two excisions complemented insc^{6}, insc^{22}, and fam^{07505} (sktl^{215}, sktl^{319}; data not shown). Because insc^{6} and insc^{22} affect insc, and fam^{07505} affects both sktl and insc, it follows that excisions that complement the insc^{6} and insc^{22} but fail to complement the fam^{07505} are potentially mutations in sktl alone. Therefore sktl^{45}, sktl^{69}, sktl^{115}, sktl^{210}, sktl^{214}, and sktl^{311} 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^{07505} 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**

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<tr>
<th></th>
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<th>Δ15</th>
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<tr>
<td>fam207505</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>insc2</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td><strong>Deletion size</strong></td>
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<td>ND</td>
<td>0.7</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Stage of lethality</strong></td>
<td>L1</td>
<td>L1</td>
<td>ND</td>
<td>L1</td>
<td>E</td>
<td>L1</td>
<td>L1</td>
</tr>
</tbody>
</table>

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 (sktlΔ15), an intermediate (sktlΔ5), and the largest (sktlΔ31) excisions. sktlΔ15 larvae are late first larval instar lethal and have no gross morphological abnormalities. Similarly, sktlΔ5 mutants are early first instar lethal and have no obvious defects. sktlΔ31 mutants are late embryonic lethal and also show no gross morphological defects as revealed by light microscopy (see also next section). sktlΔ15/ sktlΔ20 and sktlΔ5/ sktlΔ31 transheterozygotes are early first instar lethal while sktlΔ20/P49 transheterozygotes are identical in stage of lethality to sktlΔ31 homozygous embryos. While these genetic data suggest that sktlΔ15 and sktlΔ15 are strong hypomorphs and sktlΔ31 is a null allele, in situ hybridization shows that the sktl message, although severely reduced, is not completely abolished in sktlΔ20 embryos (Figure 3). These observations can be reconciled by the fact that the deletion in sktlΔ20 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.

**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 sktlΔ20 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 (Vaessen 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 Ca2+-dependent neuropeptide secretion from PC12 cells (Hay et al. 1995). In addition, several

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**Figure 3.** sktl alleles have reduced levels of sktl RNA. Panels represent lateral views of a wild-type embryo (A) and a homozygous sktlΔ20 embryo (B) at stage 11. Strong reduction in expression is seen in sktlΔ20 embryos in which a large portion of sktl is deleted, supporting the genetic evidence that suggests that sktlΔ20 is either a strong hypomorphic allele or a null mutation. Other alleles show degrees of message reduction consistent with the size of their respective deletions (not shown).
Figure 4.—Evoked excitatory junctional potentials (EJPs) in third larval bodywall 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 CaCl₂ HCl-3 solution (Stewart et al. 1994). The rapid and transient glutamatergic responses (i.e., individual EJPs) are indistinguishable between control larvae (A; sktl¹¹/TM 6 or fam⁶⁷⁵⁰⁵/TM 6) and the mutant larvae (B; sktl¹¹/fam⁶⁷⁵⁰⁵). 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 sktl¹¹/fam⁶⁷⁵⁰⁵ larvae. The resting potential was −64 mV.

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 germ-line clones using one of the excision alleles (sktl¹¹). 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 ovo¹¹ 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
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	extsuperscript{D15} and sktl	extsuperscript{D15} 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	extsuperscript{D15} and fam	extsuperscript{07505} 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	extsuperscript{D15}/fam	extsuperscript{07505} 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-

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**Table 2**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Bristle color: yellow (%)</th>
<th>n</th>
<th>Eye color: white (%)</th>
<th>n</th>
<th>Egg laying: fertile (%)</th>
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<td>1137</td>
<td>0</td>
<td>164</td>
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<td>164</td>
</tr>
<tr>
<td>D5</td>
<td>7 (1)</td>
<td>869</td>
<td></td>
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</tr>
<tr>
<td>+</td>
<td>237 (76)</td>
<td>312</td>
<td>66 (45)</td>
<td>146</td>
<td>68 (47)</td>
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Frequency of recombination between FRT sktl 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.

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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	extsuperscript{D15} 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 dav-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

PIPK5s 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; Gaiddarov et al. 1996; Salem et al. 1996; Hao et al. 1997; Martin 1997). It has also been shown that a PIP5KI is required for Ca2+-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 increasedPIP2 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; Schaefer 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 Cooly 1994). Loss of function of either protein results in bristles that lack actin filament integrity, causing bending and branching during extension (Verheyen and Cooly 1994; Cant and Cooly 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 Cooly 1994; Cant and Cooly 1996). Specifically, while the loss-of-function of sktl results in bent and wavy bristles (Figure 4A; Figure 5B and C), we did 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 of the PNS indicates that the alteration of PIP5KI levels results in female sterility. Therefore, both aspects of female sterility 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 phosphatidylinositols 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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