Sphingosine kinase is a highly conserved enzyme that catalyzes the synthesis of sphingosine 1-phosphate and reduces cellular levels of sphingosine and ceramide. Although ceramide is pro-apoptotic and sphingosine is generally growth-inhibitory, sphingosine 1-phosphate signaling promotes cell proliferation, survival, and migration. Sphingosine kinase is thus in a strategic position to regulate important cell fate decisions which may contribute to normal animal development. To facilitate studies examining the potential role of sphingosine kinase and long chain base metabolism in Drosophila development, we characterized two putative Drosophila sphingosine kinase genes, Sk1 and Sk2. Both genes functionally and biochemically complement a yeast sphingosine kinase mutant, express predominantly cytosolic activity, and are capable of phosphorylating a range of endogenous and non-endogenous sphingoid base substrates. The two genes demonstrate overlapping but distinct temporal and spatial expression patterns in the Drosophila embryo, and timing of expression is consistent with observed changes in long chain base levels throughout development. A null Sk2 transposon insertion mutant demonstrated elevated long chain base levels, impaired flight performance, and diminished ovulation. This is the first reported mutation of a sphingosine kinase in an animal model; the associated phenotypes indicate that Sk1 and Sk2 are not redundant in biological function and that sphingosine kinase is essential for diverse physiological functions in this organism.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite that provides directional cues to migrating cells (1) and exerts proliferative and anti-apoptotic effects on many cell types (2). S1P signals are transduced through both extracellular (receptor-mediated) and intracellular mechanisms. Intracellularly, S1P signaling affects cytoskeletal organization, calcium homeostasis, DNA synthesis, and the apoptotic machinery. S1P is generated by the phosphorylation of sphingosine, a reaction catalyzed by sphingosine kinase (SK). It is eliminated through dephosphorylation catalyzed by either S1P phosphatase (S1PP) or type 2 phosphatidate phosphohydrolase or through its irreversible degradation to a long chain aldehyde and ethanolamine phosphate, catalyzed by sphingosine 1-phosphate lyase (SPL) (Fig. 1).

SK is a member of a growing class of lipid kinases, including diacylglycerol kinases and phosphatidylinositol 3-kinases, that participate in cell signaling. Many studies have implicated SK activation and S1P generation in mediating angiogenesis, tumorigenicity, metastasis, cell proliferation, motility, lymphocyte trafficking, endocytosis, and survival (reviewed in Refs. 3–10). SK activation has been reported in response to many stimuli and regulates diverse functions such as heat stress response in yeast (11) and stomatal closure in Arabidopsis (12). Thus, SK activation appears to be a widely employed mechanism for propagating mitogenic and survival signals in eukaryotes. Known downstream effectors of SK activation include ERK1/2 (13), NFκB (14), and RhoA/Rho kinase (15).

SK was originally cloned in budding yeast (16), and members of the SK family have subsequently been identified in plant (17), mouse (18), rat (19), and human (20). Each has a conserved lipid kinase catalytic domain that contains the ATP-binding site. SK enzymes thus far identified each possess five conserved domains, C1 through C5 (9, 18) (Fig. 2A). These consensus sequences appear critical to the structural integrity of the proteins and probably enable substrate recognition, as they are altered in ceramide kinases (21). The SK enzymes studied to date are capable of phosphorylating erythro-sphingosine, dihydrosphingosine, and phytosphingosine in an ATP-dependent fashion and are inhibited by a growing number of compounds, including the two most well tested in biological systems, dl-threo-dihydrosphingosine (22) and N,N-dimethyl-sphingosine (23). SK activity is widely distributed in mouse and rat tissues and is primarily found in the cytosolic fraction (20, 24, 25). However, SK activity is also found to be associated with mitochondrial and microsomal fractions, and activated forms of SK are associated with the plasma membrane to which they have been demonstrated to translocate in several cell types (26, 27). Mammalian SK activity is encoded by at least two genes, Sphk1 (18, 28) and Sphk2 (29). Sphk1 is regulated at both transcriptional and post-transcriptional (phosphorylation and translocation) levels, whereas regulation of Sphk2 has not been established (see Ref. 30 and reviewed in Ref. 31). Sphk1 and -2 demonstrate distinct enzyme kinetics and tem-

Received for publication, September 26, 2003, and in revised form, January 8, 2004
Published, JBC Papers in Press, January 13, 2004, DOI 10.1074/jbc.M310647200

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This work was supported by grants from the Muscular Dystrophy Association (to G. L. H.) and National Institutes of Health Grants IR01CA77528 and IR01GM066954 (to J. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.¶To whom correspondence may be addressed: Dept. of Biology, San Diego State University, San Diego, California 92182. Tel.: 619-594-5655; Fax: 619-594-5676; E-mail: gharris@sunstroke.sdsu.edu.

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¶To whom correspondence may be addressed: Dept. of Biology, San Diego State University, San Diego, California 92182. Tel.: 619-594-5655; Fax: 619-594-5676; E-mail: gharris@sunstroke.sdsu.edu.
poral and spatial expression patterns. Additional functional distinctions between Sphk1 and Sphk2 are evident. For example, whereas Sphk1 enhances cell survival and proliferation, Sphk2 induces apoptosis through the release of cytochrome c and activation of caspase 3, potentially through a BH3 domain and Bcl-x<sub>l</sub> interactions (32). Sphk2 also appears to be the enzyme that most efficiently phosphorylates the novel immune modulator FYT720 to the active drug (33).

Cell fate must be precisely regulated during development. The formation of physiologically and morphologically normal tissues requires exquisitely choreographed developmental processes that involve proliferation, migration, and apoptosis. Because of the diverse cellular responses elicited by S1P, it is not surprising that genes involved in S1P signaling and metabolism have proven essential for the development of complex organisms (34–38). We reported previously (37) that both accumulation and depletion of sphingolipid intermediates can be achieved predictably in vivo through mutation of genes encoding sphingolipid metabolic enzymes in *Drosophila melanogaster*. Disrupting sphingolipid synthesis through mutation of ceramidase, rescue the degeneration of photoreceptor cells in *Drosophila arrestin* mutants (40). In the work presented here, we have identified two genes that encode distinct SK isoforms in *Drosophila* and have characterized a null mutation of one of these genes, *Sk2*, that results in impaired flight performance and diminished fecundity.

**EXPERIMENTAL PROCEDURES**

**Cloning of Sk1 and Sk2**—The *D. melanogaster* genomic database (www.fruitfly.org) was searched for nucleotide sequences encoding SK genes using the mouse Sphk1a sequence (GenBank™ accession number AF068748). DNA homology searches were performed via the Berkeley Drosophila Genome Project web site using the BLAST search program. Expressed sequence tags (ESTs) were identified that correspond to two Sphingosine genes, *Sk1* (CG1747) and *Sk2* (CG32484/CG2159). This nomenclature was established previously (41) and does not imply a homologous relationship between *Drosophila* SK1 and human Sphk1. The *Sk1* open reading frame was amplified by PCR from EST RE644552 using the primer pair CG1747-5' (5'-ATAGCCGCAACACAGGGAC-3') and CG1747-3' (5'-CTACTGGCCACTCTGGTCA-3') and introduced into the cloning vector pCR2.1-TOPO (Invitrogen). The *Sk1* open reading frame was subsequently recloned into yeast shuttle vector, pYES2 (Invitrogen), at HindIII and NotI restriction sites. The *Sk2* open reading frame was amplified by PCR from EST LD11247 using the primer pair CG2159-5' (5'-TCAAAGCTTGAGACAACTCCTGAT-3') and CG2159-3' (5'-TGGCTGGATCTTCTGATA-3') and cloned directly into pYES2 at HindIII and BamHI sites. The resulting galactose-inducible constructs were introduced into *Saccharomyces cerevisiae* strain JSK392 (ALC4 LYS2 ADP1) (42) using the lithium acetate method (1).

**Functional Expression and Complementation of *S. cerevisiae* Mutants**—Yeast strain JSK392 contains null alleles of endogenous SPP (DPL1), major S1PP (YSR2), and major SK (LCB4) genes (42). This strain is viable and healthy in the absence of SK activity. However, when a functional SK is expressed in this background, the cells cannot grow due to massive accumulation of LCBPs. To evaluate the ability of *Sk1* and *Sk2* to functionally complement a yeast SK mutant, strains JSK392[Sk1-pYES2] and JSK392[Sk2-pYES2] were inoculated onto uracil–agar plates containing 2% galactose and control uracil–agar plates containing 2% glucose. Complementation was demonstrated by lack of growth in the presence of galactose.

**LCBP Measurements in Yeast Overexpressing *Sk1* and *Sk2**—Endogenous yeast LCBPs were evaluated in yeast strains JSK398 (pGAL vector [LEU2] in JSK392), JSK392[Sk1-pYES2], and JSK392[Sk2-pYES2] (42) under induction and repression conditions. Colonies were picked from plates and grown overnight at 30 °C with shaking in synthetic complete medium lacking either leucine (SC-LEU) or uracil (SC-URA) in the presence of 2% glucose. One aliquot was resuspended at A<sub>600</sub> = 0.2 in rich medium containing 2% glucose (YPGal) and incubated at 30 °C with shaking (repression conditions). A second aliquot was washed three times in sterile water followed by a wash in rich media containing 2% galactose (YPGal), resuspended at A<sub>600</sub> = 0.2 in YPGal, and incubated at 30 °C with shaking (induction conditions). Cells were harvested at 12 h, and lipids were extracted essentially as described (42). The dried lipid extracts were resuspended in 2 ml of chloroform/methanol, 1:1 (v/v), and 0.5 ml of 1.5 M ammonium hydroxide in water was added while vortexing. A clear separation of the two phases was obtained following centrifugation at 1500 × g for 5 min. The aqueous phase was recovered, dried down, and resuspended in 0.5 ml of methanol/water, 1:1 (v/v), containing 0.1% acetic acid. Additional purification of the LCBPs in the aqueous phase was performed on a Strata C18-E column, as described previously (37), and the purified LCBPs were labeled with ortho-phthalaldehyde and quantified by high performance liquid chromatography (HPLC) as described previously (42).

**SK Activity in Yeast Overexpressing *Sk1* and *Sk2**—Yeast were grown essentially as described above except that overnight aliquots were resuspended at A<sub>600</sub> = 1.0 in either YPGal or YPGal and incubated at...
Drosophila Sphingosine Kinases

30 °C with shaking for 5 h. Cell extracts were prepared by vortexing with glass beads as described previously, and protein concentration was determined by using the Bradford method (Bio-Rad) (11). The substrate for the SK activity assay was prepared by drying down a mixture of LCBs and C_{16}-lysophosphatidylcholine, 1:9 (mol/mol). Substrate was resuspended in 100 mM MOPS, pH 7.2, containing 5 mM 2-mercaptoethanol by tip sonication for 20 s. Aliquots of 25 μl of substrate mixture containing 10 nmol of LCBs were added into each sample tube followed by the addition of 50 μl of 100 mM MOPS, pH 7.2, containing 5 mM 2-mercaptoethanol and 15 mM magnesium chloride, and cell extract with the equivalent of 50 μg of protein. Samples were pre-incubated for 5 min at 37 °C, and the SK reaction was started by adding 25 μl of 10 mM ATP. Samples were incubated for 30 min, at which time 1 nmol of an internal LCBP standard was added to each sample. The SK reaction was stopped by adding 0.5 ml of 1.5 M ammonium hydroxide in water and 2 ml of chloroform/methanol, 2:1 (v/v). The aqueous phase was recovered and dried down, and the LCBPs were labeled with orthophthalaldehyde and quantified by HPLC as described (42).

In Vitro Kinase Assay—Embryos aged 12–24 h were homogenized in lysis buffer and centrifuged at 100,000 × g for 60 min at 4 °C to separate membrane and cytosolic fractions. The membrane fraction (pellet) was resuspended in lysis buffer by sonication. SK activity was determined by the formation of 32P-labeled C18-S1P essentially as described (49). Relative signal intensity was determined using ImageQuant software.

In Situ Hybridization—Hybridizations were performed using standard conditions (43). Briefly, whole embryos of all stages were fixed, hybridized to a digoxigenin-labeled riboprobe (Roche Applied Science catalog number 1 201 085), and developed in a nitro

Hybridized to a digoxygenin-labeled riboprobe (Roche Applied Science catalog number 1 175 025) overnight at 45

Southern Analysis—10 μg of total RNA was electrophoresed on a denaturing 1% agarose gel, transferred to a nitrocellulose membrane, and UV cross-linked. 32P-Labeled cDNA probes were heat-denatured and hybridized to the membrane for 2 h in ExpressHyb solution (Clontech catalog number 8015-2).

Results

Identification of Two Putative D. melanogaster Sphingosine Kinases—The peptide sequence of human Sphk1 (GenBank™ accession number NP_068807) was used in TBLASTN searches against all known Drosophila sequences. Two loci with substantial sequence similarity were identified (Fig. 2A). Both were annotated as predicted genes (47) and correspond to full-length cDNA clones (48). The first, Sk1 (formerly CG1747, GenBank™ accession number AAF468045), is located on the X chromosome at region 10B13 (Fig. 2B). There are two predicted splice variants (2614 and 2472 bp, respectively) that result in alternative 5'-untranslated sequences. The predicted protein for both transcripts contains 641 amino acids and has a mass of 71 kDa. There are no reported mutant alleles of this gene. The second locus, Sk2 (formerly CG32484, GenBank™ accession number AAF47706), is located on the third chromosome at region 63A3-5 (Fig. 2C) and contains a single intron. The predicted protein product contains 661 amino acids and has a calculated molecular mass of 74 kDa. This gene has one recorded mutant allele, Sk2^KDDSD4^, which harbors an E-element insertion in the 5'-untranslated region (49).

SK1 and SK2 proteins are 39% identical and 63% similar to one another. Both proteins contain five domains conserved within the diacylglycerol kinase putative catalytic domain family. Both SK1 and SK2 are more similar to mammalian Sphk2 than to Sphhk1, with Drosophila SK1 demonstrating 33% identity and 47% similarity to murine Sphk2 versus 27% identity and 42% similarity to murine Sphk1, and Drosophila SK2 demonstrating 32% identity and 45% similarity to murine Sphk2 versus 27% identity and 39% similarity to murine Sphk1. Similarities among SK1, SK2, and the mammalian Sphk2 proteins are distributed throughout the length of the proteins and include a domain near the N terminus of ~100 amino acids shared by these three proteins but which is not present in murine or human Sphk1. A conserved SGGDG_{1,2,3}-K sphingosine kinase motif has been proposed in which the second conserved glycine is critical for ATP binding, and the conserved downstream lysine facilitates nucleotide orientation within the binding pocket (21). This motif is present in murine and human Sphk1 proteins and human Sphk2, whereas in murine Sphk2 the lysine has been replaced by an arginine. Similarly, in Drosophila SK1, the SGGDG is conserved, and an arginine replaces the lysine. However, in SK2 the motif is altered to GGDG, and an arginine is present in the 12th position, reminiscent of phosphatidylinositol 4-phosphates kinases. Murine Sphk2 was also reported recently (32) to contain a functional BH3-only domain that may be involved in the ability of this protein to induce apoptosis. Like murine Sphk2, the corresponding Drosophila SK2 domain contains 5 of 11 conserved residues of the representative motif, whereas SK1 contains only 3. The physiological relevance of these partially conserved domains is not known. Recently, variant Sphk1 and -2 proteins were identified that contain N-terminal extensions of variable lengths (14–86 residues) (33). No homologies between these N-terminal extensions and SK1 and SK2 were identified.

Sk1 and Sk2 Encode Functional Sphingosine Kinases—To determine whether these genes encode functional SK enzymes, full-length cDNA clones for Sk1 and Sk2 were sequence-verified and cloned into a galactose-inducible yeast expression vector. These constructs were then expressed in a yeast mutant strain (JSK392) deficient both in SK activity and in LCBP degradation. Endogenous yeast LCBPs accumulate to toxic concentrations when degradation is inhibited by combined mutations of both S1PP (YSR2) and SPL (DPL1). Obstructing LCBP synthesis by deletion of the major yeast SK gene LCBA in a triple knockout strain prevents this toxicity, whereas expression of a functional SK gene in the triple knockout background induces toxicity (42). Expression of either Sk1 or Sk2 in this background provides functional
complementation (Fig. 3A). The resulting growth inhibition was more accurately quantified as a function of time in liquid media (Fig. 3B). Sk1 and Sk2 inhibited growth in liquid media containing galactose (Fig. 3B) but not glucose (data not shown). Upon induction of Sk1 or Sk2 expression, C_{18}-phytosphingosine 1-phosphate and C_{18}-dihydrosphingosine 1-phosphate accumulate markedly, demonstrating that SK1 and SK2 are capable of phosphorylating endogenous yeast LCBs in vivo (Table I).

Biochemical Characteristics of SK1 and SK2—To determine the substrate specificities of SK1 and SK2, a variety of substrate combinations was evaluated in vitro. As shown in Table II, both Drosophila SK enzymes demonstrated activity against C_{14} sphingosine (the predominant Drosophila-free LCB (46)) as well as dihydro- and phytosphingosines of various chain lengths. SK activity was predominantly localized to the cytosol (Fig. 5A), which contains 3.2-fold higher activity against a C_{18} sphingosine substrate than the membrane fraction.
**TABLE I**

**Sphingolipid content of yeast overexpressing Drosophila SK**

| Culture                  | C_{16}DHS-IP | C_{16}PHS-IP |
|--------------------------|--------------|--------------|
| JSK392                   | ND           | ND           |
| JSK392 with [pYES-Sk1]   | 26.9 ± 7.9   | 6.6 ± 2.8    |
| JSK392 with [pYES-Sk2]   | 111.9 ± 41.3 | 9.7 ± 4.3    |

*Embryonic Expression of Sk1 and Sk2 mRNA Is Spatially and Temporally Regulated*—Developmental expression of Sk1 and Sk2 mRNA was assessed by *in situ* hybridization of wild-type embryos (Fig. 4A). Transcripts from both genes are present in the syncytial blastoderm (stage 4) and decline shortly thereafter. This is followed by overlapping but distinct expression patterns for the two genes. Sk1 demonstrates two waves of expression. The first begins at stage 6, peaks at stage 10–11, and is localized to the anterior and posterior midgut rudiment (endoderm) and the mesoderm. The staining in the posterior midgut appears more punctate in an Sk2 null background, possibly due to a decrease in the background staining (predicted to be high due to the sequence similarity between the two genes). Although this pattern resembles that of primordial germ line cells (PGCs), the Sk1-positive cells did not coincide with those expressing VASA, a PGC-specific marker (44) (Fig. 4B). Sk1 expression subsequently declines but persists ubiquitously in the embryo, with the highest level maintained in the gut. The second, stronger wave occurs throughout the midgut and hindgut epithelium during stage 16 and beyond.

Early in embryogenesis, expression of Sk2 is similar to Sk1, appearing in the endoderm and mesoderm but declining earlier (approximately at stage 9). Sk2 demonstrates persistent, strong expression in the salivary glands upon formation of the primordial tissue at stage 11 and transient expression in the hindgut during stage 14. Notably, there is a period of significant mesodermal expression at stage 13 during which myoblasts fuse to form muscle fibers. The absence of staining in the 

**Sk2<sup>KG05894</sup>** insertion line demonstrates probe specificity and confirms that Sk2 transcription is disrupted.

Real time, quantitative PCR was performed to quantitate the global expression of Sk1 and Sk2 (Fig. 5B). Sk1 expression remains low in early embryos until 12 h post egg deposition (approximately at stage 15). Expression then increases, peaks in the early pupa, and remains elevated in the adult. Sk2 expression is highest in the early embryo and then declines until the early pupa. Sk2 expression is severalfold higher than Sk1 at all stages of the life cycle as determined by quantitative PCR and Northern analysis (data not shown), although absolute quantitation was not evaluated. Both genes are more highly expressed in adult males than in adult females.

The temporal expression pattern of these genes agrees with the profile of accumulated sphingolipids. In wild-type Canton-S (CS) flies, there is low, basal accumulation of C₁₄ LCBs (Fig. 5C), the predominant class of metabolites in *Drosophila* (50). LCB levels become markedly elevated in the absence of Sk2 activity (see below).

*Identification of an Sk2 Mutant*—In order to identify the biological significance of *Drosophila* SK, existing Sk1 and Sk2 mutant lines were sought. Although no Sk1 mutant was available, the insertion line Sk2<sup>KG05894</sup> was identified. This line harbors a modified P-element in the 5’-untranslated region of Sk2, 54 nucleotides upstream of the start codon, strongly suggesting that this was a molecular “null” mutation. Lack of Sk2 expression was confirmed both by *in situ* hybridization (Fig. 4A) and Northern blot analysis (Fig. 6A). Precise excision of the P-element (Sk2<sup>53A</sup>) restores transcription of Sk2. Consistent with the role of Sk2 in LCB metabolism, there is a marked increase in sphingolipid content in the Sk2 mutant line at all stages of the life cycle (Fig. 5C). LCB accumulation is concordant with the Sk2 pattern of expression in that the greatest difference in Sk2 mutants versus wild type is found in the early embryo when wild-type Sk2 expression is at its highest level. Thus, Sk2 plays a key role in the regulation of LCBs during development.

**Sk2 Mutants Are Defective in Flight Behavior and Reproduction**—In order to determine whether SK is required for normal
biological functions in vivo, a homozygous Sk2 mutant was examined for anatomical, developmental, and behavioral phenotypes. Sk2<sup>KG05894</sup> flies are homozygous viable with no gross external morphological defects. There is no reduction in viability at any stage in the life cycle, and adult longevity is normal (data not shown). However, behavioral analysis uncovered a consistent reduction in flight ability (Fig. 6B). Evaluation of the morphology and patterning of the indirect flight muscles that power flight did not reveal any structural defects that could explain this phenotype (data not shown). The impaired flight performance was fully rescued by precise excision of the P-element.

Consistent with other known mutations of sphingolipid metabolic enzymes (37), Sk2<sup>KG05894</sup> homozygotes have reproductive defects. The number of eggs produced by mutant females is 43% that produced by wild-type controls (Fig. 6B). The eggs, however, are viable and develop normally. The decrease in fecundity of Sk2<sup>KG05894</sup> is fully rescued by precise excision of the P-element (Fig. 6B). Dissection of adult Sk2<sup>KG05894</sup> females revealed no evidence of morphological defects of the ovaries and

**Table II**

*In vitro phosphorylation of sphingolipid substrates*

Cultures were started in galactose media at A<sub>600</sub> = 1.0 and induced for 5 h before harvesting. Lysates were incubated with one of two substrate mixtures: A, C<sub>1<sub>4</sub>/C<sub>1<sub>6</sub>/C<sub>1<sub>8</sub>Soc; B, C<sub>1<sub>6</sub>/C<sub>1<sub>8</sub>DHS, or C<sub>1<sub>8</sub>PHS. Phosphorylated products were quantified by HPLC. Values represent pmol/×10<sup>6</sup> cells × min ± S.D. ND, not detectable.

| Substrate mixture A | JSK392 | JSK392 with [pYES-Sk1] | JSK392 with [pYES-Sk2] |
|---------------------|--------|------------------------|------------------------|
| C<sub>1<sub>4</sub>S1P | ND     | 3.9 ± 1.2              | 18.8 ± 3.4             |
| C<sub>1<sub>6</sub>S1P | ND     | 2.1 ± 0.6              | 13.4 ± 0.9             |
| C<sub>1<sub>8</sub>S1P | ND     | 3.0 ± 0.3              | 7.8 ± 0.4              |
| Total S1P           | ND     | 9.0 ± 2.1              | 40.0 ± 4.7             |
| Substrate mixture B |        |                        |                        |
| C<sub>1<sub>6</sub>DHS-1P | ND   | 7.3 ± 0.6              | 19.7 ± 1.7             |
| C<sub>1<sub>8</sub>DHS-1P | ND   | 6.9 ± 1.1              | 16.5 ± 1.6             |
| Total DHS-1P        | ND     | 14.2 ± 1.7             | 36.2 ± 3.3             |
| C<sub>1<sub>8</sub>PHS-1P | ND   | 3.8 ± 0.4              | 5.9 ± 0.7              |

**Fig. 4.** *In situ* localization of Sk1 and Sk2 mRNA during embryogenesis. A, both transcripts are present in the syncytial blastoderm suggesting maternal contribution. Sk1 and Sk2 expression subsequently decline until gastrulation at which time they both appear in the mesoderm and endoderm. Expression again declines at stage 11 for Sk1 and stage 9 for Sk2. At stage 12–13 weak but detectable expression of Sk1 can be found throughout the embryo, predominantly in the midgut (arrow) and hindgut (arrowhead). Persistent expression of Sk2 in the salivary glands begins at this stage (arrow), and transient expression is noted in the mesoderm (arrowhead). In the late embryos (stage 16–17) expression of Sk1 is strong in the midgut and hindgut, and Sk2 is seen throughout the gut but predominantly in the salivary glands. Sk2 staining is undetectable in Sk2<sup>KG05894</sup> mutants. This mutant background gives a more punctate staining pattern for Sk1 in the stage 9 endoderm (arrow). B, posterior midgut rudiment of a stage 9 Sk1<sup>KG05894</sup> homozygote stained for Sk1 mRNA (red) and VASA protein (green). The arrow indicates a primordial germ-line cell expressing Sk1. Arrowheads indicate non-primordial germ-line cells expressing Sk1.
Fig. 5. Expression of Sk genes. A, cell lysates from wild-type embryos were separated into membrane and cytosolic fractions and then assessed for SK activity based on their ability to phosphorylate C14 sphingosine with [γ-32P]ATP. Phosphorylated LCBs were isolated by thin layer chromatography and visualized by autoradiography. Signal intensity was quantified with ImageQuant software and depicted graphically and numerically. B, relative expression of the indicated genes in unfertilized eggs, staged embryos, 1st instar and 3rd instar larvae, early and late pupae, and adult females and males was assessed by real time quantitative PCR. Expression was normalized to a ribosomal protein subunit gene (Rpl32), standardized to expression in unfertilized eggs, and plotted graphically. C, quantification of total C14 LCBs of staged wild-type (CS) and Sk2KG05894 embryos (Sk2). Numbers in parentheses represent fold increase of Sk2KG05894 over CS.

no abnormal masses other than an increase in fatty tissue that normally surrounds the gonads. The ovaries of Sk2KG05894 mutants, however, consistently appear enlarged compared with wild-type controls due to an increase in the number of mature eggs (Fig. 6, D–G). This was considered an egg-retention phenotype, as diminished fecundity and egg laying were observed with no evidence of altered oocyte survival in mutants compared with wild-type flies (data not shown). 68% of the ovaries from Sk2KG05894 females exhibited the egg-retention phenotype (n = 88) compared with 8% of wild-type ovaries (n = 66). This ovulation defect was present in Sk2KG05894 females when crossed to wild-type males and absent in wild-type females crossed to Sk2KG05894 males.

DISCUSSION

This report characterizes two SK enzymes of D. melanogaster and describes their involvement in regulating LCB metabolism during development, as well as physiological consequences resulting from the loss of function of Sk2. Like the putative Caenorhabditis elegans SK, Drosophila SK1 and SK2 show greater resemblance to mammalian Sphk2 than to Sphk1, suggesting that Sphk2 is the more primitive of the two mammalian enzymes. Because there is no evidence that Sphk2 expression or activity are regulated, the ability of Sphk1 to be activated in response to physiological stimuli may be a relatively recent evolutionary step, coordinating S1P signaling with other growth regulatory pathways, thus allowing potentiation and fine-tuning of critical mitogenic and migration signals.

The functional complementation of the yeast mutants and in vitro kinase activity of the yeast cell lysates verify that these genes encode functional SK enzymes. Both kinases have broad substrate specificities as shown by their ability to phosphorylate a range of substrates from 14 to 18 carbons in length. Notably, shorter chain length LCBs (C14–C16) are the only identified endogenous LCB species in Drosophila, which contrasts with the C18 LCBs found in mammalian cells (46, 50). This is interesting in light of the recent finding that the generation of palmitate (but not lauric acid or myristic acid) by this pathway is responsible for the regulation of SREBP cleavage (and consequently fatty acid synthesis) in Drosophila (41, 51). Although the explanation for this is not clear, it is possible that the endogenous versus exogenous substrates used in different experiments may be responsible for these observations.

SK activity in Drosophila is predominantly localized to the cytosolic fraction. This is consistent both with the absence of strong consensus transmembrane motifs in either protein product and with previous reports describing the localization of mammalian SK activity (9, 52).

During embryogenesis, expression of Sk1 and Sk2 is highest in the developing gut (Fig. 4A). This pattern is also common to Sply (37, 53) and lace (39, 53) reflecting the functional relationship among these genes and suggesting the potential for coordinate gene regulation. Gut localization of SPL is also observed in C. elegans (38), suggesting that in simple metazoans gut tissues may comprise the major sites of sphingolipid synthesis and clearance for the whole organism. The peak in expression of both genes in the early pupa (Fig. 5B) suggests a requirement of sphingolipid catabolism for tissue reorganization during metamorphosis.

Phosphorylation of LCBs is required for the generation of S1P, a well characterized signaling molecule. In addition, this phosphorylation is a prerequisite for the degradation of LCBs. LCB accumulation is 1.8-fold higher in Sk2KG05894 homozygous adults relative to wild-type controls, indicating that Sk1 cannot entirely compensate for loss of Sk2 expression. However, this compares to a 10-fold increase in LCBs in SplyKG05894 homozygotes, suggesting that significant SK activity and LCB degradation must still exist in the Sk2 mutant. Sk2 activity is re-
FIG. 6. Analysis of an Sk2 mutant. A, Northern analysis of RNA from adult flies indicates that Sk2 is not expressed in Sk2KG05894 flies, but transcription is restored by precise excision of the P-element. B, Sk2KG05894 adults have a 20% reduction in flight performance and a 57% reduction in fecundity. Both of these defects are corrected by excision of the P-element. C, schematic of Drosophila ovaries. Each ovary consists of a bundle of tubular ovarioles. Meiosis occurs in the gerarium. As the oocyte develops it progresses through its ovariole toward the oviduct. Mature oocytes enter the oviduct (ovulation) and are fertilized with sperm stored in the seminal receptacle before deposition. D-G, ovaries were dissected from 5-day-old adult females of the indicated genotype and photographed under ×32 magnification. D, Canton-S female mated to Canton-S male. E, Sk2KG05894 female mated to Sk2KG05894 male. F, Canton-S female mated to Sk2KG05894 male. G, Sk2KG05894 female mated to Canton-S male.
quired to maintain regulated levels of LCBs throughout development. Disruption of Sk2 activity leads to LCB accumulation throughout the fly life cycle. The dynamics of this regulation become apparent when one observes the effect of Sk1 expression in the Sk2 mutant. The greatest accumulation of LCBs is observed in the Sk2 mutant early in embryogenesis when Sk1 expression is low and Sk2 expression is normally at its highest level. As Sk1 expression increases later in embryogenesis (after 12 h), LCB content of the Sk2 mutant begins to approach wild-type levels. The normal developmental increase in LCB accumulation in wild-type pupae (1.7-fold over larvae) is exaggerated in the Sk2 mutant (3.4-fold). The reducency of Sk1 and Sk2 function is most apparent in adult flies because Sk2 mutants have the lowest fold increase of LCBs (1.8-fold) over wild-type controls at this stage.

The Sk2 phenotypes are similar to, but less severe than, those seen in Spyl mutants. Disruption of either gene results in diminished flight performance and fecundity. The reduction of fecundity may be explained, in part, by the retention of mature eggs in the ovaries of Sk2 mutants, a phenotype that is also observed in Spyl mutants. This phenomenon is similar to the egg retention seen in wild-type un-mated females (data not shown) raising the possibility that Sk2

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