Identification and characterization by electrospray mass spectrometry of endogenous *Drosophila* sphingadienes

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Abstract Sphingolipids comprise a complex group of lipids concentrated in membrane rafts and whose metabolites function as signaling molecules. Sphingolipids are conserved in *Drosophila*, in which their tight regulation is required for proper development and tissue integrity. In this study, we identified a new family of *Drosophila* sphingolipids containing two double bonds in the long chain base (LCB). The lipids were found at low levels in wild-type flies and accumulated markedly in *Drosophila Sply* mutants, which do not express sphingosine-1-phosphate lyase and are defective in sphingolipid catabolism. To determine the identity of the unknown lipids, purified whole fly lipid extracts were separated on a C18-HPLC column and analyzed using electrospray mass spectrometry. The lipids contain a LCB of either 14 or 16 carbons with conjugated double bonds at C4,6. The $\Delta^{4,6}$-sphingadienes were found as free LCBs, as phosphorylated LCBs, and as the sphingoid base in ceramides. The temporal and spatial accumulation of $\Delta^{4,6}$-sphingadienes in *Sply* mutants suggests that these lipids may contribute to the muscle degeneration observed in these flies.—Fyrst, H., X. Zhang, D. R. Herr, H. S. Byun, R. Bittman, V. H. Phan, G. L. Harris, and J. D. Saba. **Identification and characterization by electrospray mass spectrometry of endogenous *Drosophila* sphingadienes. *J. Lipid Res.* 2008.** 49: **597–606.**

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Sphingolipids are a diverse group of membrane lipids that are highly conserved throughout evolution (1, 2). In mammalian cells, sphingolipid structure, composition, and metabolism have been well characterized. Knowledge of sphingolipid structure has facilitated in-depth analyses of the contribution of sphingolipids to membrane organization and their function in signal transduction events and normal physiology. Such studies have defined an important role for higher order sphingolipids in the formation of membrane subdomains (lipid rafts) wherein growth factor signaling and recruitment occur (3–6), and sphingolipid metabolites have been shown to participate in signaling pathways regulating the key processes of cellular proliferation, migration, stress responses, programmed cell death, angiogenesis, and immune cell trafficking (7–12).

We have been exploring the physiological roles of sphingolipids in the genetically tractable organism, *Drosophila melanogaster*. In this species, tight regulation of sphingolipid levels is required for proper development, reproduction, and the maintenance of tissue integrity, as demonstrated by the severe phenotypes observed in mutants with disrupted sphingolipid metabolism (13–17). However, a clear understanding of the role of sphingolipid metabolism and, in particular, the mechanisms by which sphingolipid metabolites influence physiological processes in this organism has been hampered by an incomplete knowledge of the chemical structures of endogenous *Drosophila* sphingolipids and their metabolic products.

Previously, we found that the *Drosophila* free sphingoid bases or long chain bases (LCBs) are composed largely of C_{14}- and C_{16}-sphingosine and dihydrosphingosine (18). In the current study, we have identified a second family of sphingolipids recognized by their differential separation on HPLC compared with known *Drosophila* sphingolipid species. Mass spectrometry approaches were used to characterize the structures of these unknown lipids as C_{14}- and C_{16}-sphingadienes with Δ-4,6 conjugated double bonds and to further identify a family of related lipids built upon the same $\Delta^{4,6}$-sphingadiene LCB. The presence of sphingolipids containing a $\Delta^{4,6}$-sphingadiene LCB has been reported in other insect species. A study of sphingomyelins from the tobacco hornworm *Manduca sexta* revealed the

Abbreviations: dsRNA, double-stranded RNA; LCB, long chain base; LCBP, long chain base phosphate; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; RNAi, RNA interference; SPL, sphingosine-1-phosphate lyase.

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presence of $\Delta^{4,6}$-sphingadiene LCBs in sphingomyelin, ceramide-phosphoethanolamine, and ceramide (19). Moreover, a ceramide compound purified from larvae of the silk moth *Bombyx mori* was shown to contain a $\Delta^{4,6}$-sphingadiene LCB (20). In both studies, the $\Delta^{4,6}$-sphingadiene-containing ceramides were found to have potent although diverse biological effects. The ceramide compound from *M. sexta* was found to increase apoptosis in an embryonic *M. sexta* cell line (19), whereas the compound from *B. mori* was shown to promote neurite outgrowth in the rat pheochromocytoma cell line PC12 (20).

Sphingosine-1-phosphate lyase (SPL) is an important enzyme in the sphingolipid degradation pathway, as it catalyzes the cleavage of a long chain base phosphate (LCBP) to yield a long chain aldehyde and ethanolamine phosphate, thereby irreversibly removing LCBPs from the sphingolipid pool. We previously reported the severe reproductive organ and muscle phenotypes of the *Drosophila* SPL mutant fly *Sply* and showed increased levels of sphingosine, dihydrosphingosine, and the corresponding LCBPs in the adult *Sply* fly (15). In this study, we demonstrate the presence of *Drosophila* sphingolipids containing $\Delta^{4,6}$-sphingadiene LCBs. These lipids are found in wild-type flies from mid embryogenesis to adulthood and accumulate markedly in the *Sply* mutant. Although we found a general accumulation of total LCBs and ceramides in all of the tissues of *Sply* mutant flies, a greater accumulation of $\Delta^{4,6}$-sphingadienes was found in the thorax, which contains the flight muscles that undergo spontaneous degeneration. Moreover, we found that $\Delta^{4,6}$-C_{14}-sphingadienes and the corresponding C_2-sphingadiene-ceramide decreased cell proliferation in the *Drosophila* wing disc cell line Cl.8. These findings suggest that $\Delta^{4,6}$-sphingadiene-containing sphingolipids likely contribute to flight muscle tissue degeneration in the *Sply* mutant.

EXPERIMENTAL PROCEDURES

Drosophila stocks

Wild-type Canton-S (BL-1), SPL knockout strain $Sply^{05091}$ (BL-11393), and $lace^{2/k05305}$ (BL-3156 and BL-12176) were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). Flies were reared on standard fly medium at room temperature. Flies carrying the double knockout mutation *Sply/lace* and the *Sply* revertant $Sply^{14a}$ were generated as described previously (15). $Sk2^{KG05894}$ was a gift of the P-element Screen/Gene Disruption project of the Bellen/Rubin/Spradling laboratories (14). In all cases, control and mutant flies were reared in parallel under identical conditions. For developmental analysis, adult flies were allowed to deposit embryos on grape juice agar plates, and embryos were collected, staged, and prepared as described (18). For the isolation of tissue, 100 female flies, 4 to 6 days old, were euthanized, and abdomen, head, thorax, and ovaries were collected.

Cell cultures

Drosophila S2 cells and Cl.8 cells were obtained from the *Drosophila* Genomics Resource Center at Indiana University. S2 cells were cultured at 28°C in Schneider's medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely passaged every second day to a density of $\sim 2 \times 10^6$ cells/ml. Cl.8 wing disc cells were cultured at 28°C in Shields and Sang Medium 3 (Sigma-Aldrich, St. Louis, MO) supplemented with 2% heat-inactivated FBS, 12.5 IU/100 ml insulin, and 2.5% extract from adult wild-type flies.

Preparation of double-stranded RNA

A DNA fragment containing a 720 bp segment of the *Sply* open reading frame containing T7 promoter sequences at the 5′ and 3′ ends was generated by PCR using Vent DNA polymerase (New England Biolabs, Beverly, MA). As a control, a DNA fragment containing the full-length open reading frame of an unrelated *Caenorhabditis elegans* gene, *elt2,* was generated using similar methods. The preparation of double-stranded RNA (dsRNA) in vitro was performed in a reaction mixture of diethylpyrocarbonate-treated water containing 2 μg of DNA template, 10 mM ribonucleotide triphosphate, 150 units of T7 polymerase enzyme (New England Biolabs), and 8 μl of 10× T7 polymerase buffer in a total volume of 80 μl. Samples were incubated at 37°C for 8 h, the reaction was stopped, and the RNA was precipitated by adding 8 μl of 3 M sodium acetate, pH 5.2, and 200 μl of 2-propanol. Samples were stored at −20°C overnight, and the precipitate containing the RNA was isolated by centrifugation at 14,000 *g* for 30 min. The supernatant was discarded, and the pellet was washed twice with 200 μl of 70% ethanol and then allowed to air-dry for 10 min. The pellet was then dissolved in 50 μl of diethylpyrocarbonate-treated water, and annealing of the two RNA strands was performed by heating the sample for 5 min at 75°C followed by slow cooling to room temperature. The status of the synthesized dsRNA was evaluated on a 1% agarose gel.

RNA interference experiment

S2 cells were harvested and resuspended in serum free Schneider's *Drosophila* medium to a density of 1×10^6 cells/ml. The *Sply* or control dsRNA was then added directly to the cell suspension to a final concentration of 15 μg dsRNA/ml, and cells were incubated at room temperature for 30 min. After incubation, 2 volumes of Schneider's *Drosophila* medium containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin was added. For each RNA interference (RNAi) experiment performed, the dsRNA treatment was repeated on 2 consecutive days.

Synthesis of $\Delta^{4,6}$-C_{14}-sphingadiene and the corresponding C_2-sphingadiene-ceramide

The $\Delta^{4,6}$-sphingadiene backbone was prepared according to a procedure described previously (21). *N*-Acetylation of sphingadiene to generate (2*S*,3*R*,4*E*,6*E*)-2-acetamidotetradecadiene-1,3-diol (C_2-sphingadiene ceramide) was carried out with *p*-nitrophenyl acetate followed by purification using flash chromatography (gradient of chloroform-methanol, 15:1 to 9:1). The purity and fragmentation patterns of the final products were verified by electrospray ionization mass spectrometry. Samples were scanned from *m/z* 100 to 350 in positive mode on a Micromass Quattro LCZ (Waters Corp., Milford, MA).

Preparation of *Drosophila* lipid extracts

Samples containing 50 mg of frozen intact fly material or isolated fly tissues were placed in a glass Potter Elvehjem homogenizer. Twenty microliters of an internal standard mixture containing 200 pmol of each C_{17}-sphingosine (Avanti Polar Lipids, Alabaster, AL) and C_{17}-sphingosine-1-phosphate

(Matreya, Inc., Pleasant Gap, PA) was then added, and fly materials were homogenized in 0.5 ml of methanol until the pestle moved smoothly. An equal volume of water was then added, and homogenization was continued with another 10 strokes. Fly homogenates were transferred to a glass tube, and a two-phase separation was obtained after the addition of 1 ml of chloroform and 0.75 ml of 1 M ammonium hydroxide followed by vortexing and a 10 min spin at 1,000 *g*. For the analysis of LCBPs, the water phase was recovered, dried down, and resuspended in 0.1 ml of methanol-water (1:1, v/v). For the analysis of LCBs, a portion of the organic phase was recovered, dried down, and resuspended in 0.1 ml of methanol. For the analysis of ceramides, a portion of the organic phase was recovered, dried down, and resuspended in 0.5 ml of 1 M potassium hydroxide in methanol, and ceramides were hydrolyzed by incubating for 1 h at 90°C. After hydrolysis, a two-phase separation was obtained as described above and the organic phase was recovered.

Solid-phase extraction and HPLC analysis

Lipid extracts were purified on a Strata C18-E solid-phase extraction column, and LCBs were derivatized with *ortho*-phthalaldehyde and separated by HPLC as described (18).

Mass spectrometry analysis

For the identification of the structure of novel *Drosophila* LCBs, the Strata C18-E purified lipid extract was separated on a C18-HPLC column (2.0 × 50 mm; S-5 120 Å) (Waters Corp.) at a flow rate of 0.4 ml/min. The gradient used was from 30% to 80% methanol containing 0.1% formic acid in 10 min and 80–95% methanol containing 0.1% formic acid in 2 min. The data were acquired in positive mode on an Esquire$_{3000}^{plus}$ ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Data acquisitions with up to AutoMS^4 mode were applied for the characterization of unknowns. Crude *Drosophila* lipid extracts prepared as described above were used for the quantitation of LCBs, LCBPs, and ceramides. Lipids were separated on a C18-HPLC column (2.0 × 75 mm; Luna) (Phenomenex, Torrance, CA) at a flow rate of 0.25 ml/min. The gradient used was from 50% to 80% methanol containing 0.1% formic acid in 4 min and 80% methanol containing 0.1% formic acid for 6 min. Intact ceramides were analyzed after direct injection of 10 μl of the organic phase from the lipid extraction described above. The mobile phase was 95% methanol containing 0.1% formic acid. The flow rate was 0.05 ml/min. The data were acquired in positive mode on a Micromass Quattro LCZ (Waters Corp.). Lipids were identified based on their specific precursor and product ion pair and quantitated using multiple reaction monitoring as described (22).

Treatment of *Drosophila* Cl.8 cells with $\Delta^{4,6}$-C_{14}-sphingadiene and the corresponding C_2-sphingadiene-ceramide

Drosophila Cl.8 cells were treated for 6 h with 20 μM $\Delta^{4,6}$-sphingadiene-containing lipids in the presence of 2% heat-inactivated FBS.

Cell viability

The viability of the cells was determined by measuring their ability to hydrolyze the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into formazan according to a standard procedure using the CellTiter 96 Aqueous NonRadioactive Cell Proliferation Assay (Promega, Madison, WI). Cl.8 cells were seated on a 96-well plate and lipid-treated in place. Twenty microliters of MTS substrate was added to each well, and the 96-well plate was incubated at 28°C.

RESULTS

Sply mutants demonstrate myopathy and ovarian degeneration

SPL catalyzes the final step in sphingolipid degradation, namely the cleavage at $C_{2\text{-}3}$ of LCBPs. The *Drosophila Sply* mutant contains a P-element transposon insertion within the open reading frame of the SPL gene, resulting in a complete lack of expression of the SPL homolog SPLY. Flies homozygous for this mutation undergo proper embryogenesis and emerge from pupation. However, female homozygotes demonstrate diminished fertility, which is associated with a progressive loss of ovarian tissue as a result of apoptotic cell death (17). In addition, adult homozygotes exhibit a flightless phenotype, attributable to a myopathy resulting from degeneration of the dorsal longitudinal muscles that power the wings (15).

Sphingolipid intermediates are responsible for *Sply* mutant phenotypes

Previously, we demonstrated that the ovarian degeneration and muscle wasting were not observed in homozygous *Sply* revertants *Sply*^{14a} (15, 17). This finding confirms that the loss of *Sply* expression is responsible for the observed phenotypes. The *Sply* phenotypes were also ameliorated by the introduction of a second mutation in the *lace* gene, which encodes one subunit of serine palmitoyltransferase, the first enzyme in the sphingolipid biosynthetic pathway (15). This suggested that the phenotypes of the *Sply* mutant are caused by abnormal accumulation of sphingolipid intermediates, a state that is corrected by reducing the synthesis of these lipids. In support of this possibility, we found that the predominant LCBs in the fly, which we had previously determined to be saturated dihydrosphingosine and Δ-4 monounsaturated sphingosines with 14 and 16 carbon chains (15, 18), were markedly increased in *Sply* mutants but normalized in the double mutant cross. However, in analyzing the sphingolipids in our fly extracts, we noticed the presence of an unknown lipid, which was also increased in the *Sply* mutant and normalized in the double mutant cross. This unknown lipid was not initially recognizable and was thus referred to as "lipid X."

Identification of a novel *Drosophila* sphingolipid species by HPLC

Lipid X was readily detected in our HPLC system after derivatization with *ortho*-phthalaldehyde, which modifies free amino groups. Lipid X eluted from the HPLC system with a different retention time than C_{14}-sphingosine and C_{14}-sphingosine-1-phosphate standards when tested under different running conditions (**Table 1**). By changing the pH of the potassium phosphate buffer from 7.2 to 5.5, a 3.3 min shift in the retention time of the C_{14}-sphingosine-1-phosphate standard was observed. This change in pH

TABLE 1. Retention time of *ortho*-phthalaldehyde-derivatized LCBs and LCBPs using different HPLC mobile phases

Mobile Phase	C_{14}-Sphingosine-1-Phosphate Standard	C_{14}-Sphingosine Standard	C_{14}-Sphingosine from Fly Extract	Lipid X from Fly Extract
		min		
A				
Methanol/water/1 M TBAP (82:17:1)	14.8	19.1	19.0	14.8
Methanol/water/1 M TBAP (79:20:1)	22.5 (7.7)	27.3 (8.2)	27.1 (8.1)	22.1 (7.3)
B				
Methanol/10 mM potassium phosphate/1 M TBAP (81:18:1), pH 7.2	15.0	21.4	21.8	17.1
Methanol/10 mM potassium phosphate/1 M TBAP (81:18:1), pH 5.5	18.3 (3.3)	21.9 (0.5)	22.0 (0.2)	17.2 (0.1)

LCB, long chain base; LCBP, long chain base phosphate; TBAP, tetrabutylammonium dihydrogen phosphate. Values shown are averages of at least three independent measurements. Numbers in parentheses represent the minute change in retention time obtained by changing the percentage of methanol (mobile phase A) or the pH of the potassium phosphate buffer (mobile phase B).

had no effect on the retention time of lipid X, indicating that this lipid is not phosphorylated.

As shown in **Table 2**, lipid X was significantly increased in the *Sply* mutant compared with wild-type controls. To a lesser degree, lipid X was increased in a null *Sk2* transposon insertion mutant that has reduced capacity to phosphorylate LCBs as a result of the loss of expression of one of two sphingosine kinases present in this organism. The *Sk2* mutant demonstrates impaired flight performance and reproductive defects that are less severe than those observed in the *Sply* mutant. In contrast, lipid X was barely detectable in the *lace* mutant, which is defective in sphingolipid synthesis. Lipid X levels were reduced to wild-type levels in the *Sply* revertant line $Sply^{14a}$, which shows minimal if any of the defects observed in the *Sply* mutant. Lipid X levels in the *Sply/lace* double mutant were intermediate between those of the *Sply* mutant and the wild type. These double mutants demonstrate correction of the *Sply* mutant muscle dropout but exhibit impaired flight performance, similar to the *Sk2* mutant. Based on these findings, we hypothesized that lipid X was a sphingolipid and most likely a free LCB and that the level of lipid X accumulation correlates with the presence and severity of flight impairment, reproductive defects, and tissue degeneration.

Characterization of lipid X by mass spectrometry

By LC-MS analysis, lipid X was found to have a molecular weight of 241.1. Because the molecular weight found for C_{14}-sphingosine is 243.1 and the molecular weight found for C_{14}-dihydrosphingosine is 245.1, we suspected that lipid X was a C_{14}-LCB with two double bonds. **Figure 1** shows a LC-MS-ESI+ run of lipids extracted from adult flies, identifying LCBs in wild-type and *Sply* mutant flies. In addition to lipid X at *m/z* 242.1, a peak at *m/z* 270.1 was identified, suggesting the presence of the C16 species. To verify the structure of lipid X and map the location of the double bonds, an LC-MS spectrum of lipid X was obtained and compared with that of C_{14}-sphingosine. As shown in **Fig. 2A**, the peak intensity of intact lipid X (*m/z* 242.1) was much lower than that of its water-loss product ion (*m/z* 224.0). In comparison, the peak intensity of the C_{14}-sphingosine (*m/z* 244.1) was only slightly lower than that of its water-loss product ion (*m/z* 226.1) (Fig. 2B). The stability of lipid X's water-loss product suggested that the second double bond was conjugated with the first double bond. The fragmentation pattern of the carbon chain of the water-loss component of lipid X (*m/z* 224.0) was further investigated by AutoMS^n up to 3 using an LC-ESI ion trap MS system (**Fig. 3**). As shown in Fig. 3A, the product ion at *m/z* 67 was barely detected, whereas that at *m/z* 93 was the most abundant product ion. Comparison of the MS^3 spectra of lipid X minus water (*m/z* 224.0) and C_{14}-sphingosine minus water (*m/z* 226.1) indicated that the second double bond was located between carbons 6 and 7, thereby identifying lipid X as a $\Delta^{4,6}$-C_{14}-sphingadiene. The fragmentation pattern obtained for the endogenous $\Delta^{4,6}$-C_{14}-sphingadiene was compared with the fragmentation pattern obtained for our synthesized $\Delta^{4,6}$-C_{14}-sphingadiene standard (see Experimental Procedures), and they were found to be identical (data not shown).

Identification of $\Delta^{4,6}$-sphingadiene-containing sphingolipids in *Drosophila*

The presence of the $\Delta^{4,6}$-sphingadiene backbone was sought in other *Drosophila* sphingolipids. $\Delta^{4,6}$-Sphingadiene was found to contribute to the structure of LCBPs and ceramides of both C14 and C16 chain length using ESI-MS/MS. There was no difference in the profile of molecular species of ceramide containing the $\Delta^{4,6}$-sphingadiene backbone compared with those containing a sphingosine backbone. The $\Delta^{4,6}$-sphingadiene backbone was also detected in ceramide-phosphoethanolamine (data not shown). These

TABLE 2. HPLC analysis of endogenous lipid X in adult flies from different *Drosophila* strains

Lipid	Canton-S	$Sply^{05091}$	$lace^{2/k05305}$	$Sply^{14a}$	$Sply^{05091}/lace^{2/k05305}$	$Sk2^{KG05894}$
Lipid X (pmol/mg protein)	9.04 ± 2.95	63.83 ± 4.41	0.82 ± 0.41	9.92 ± 2.34	40.63 ± 6.74	43.41 ± 4.38

Values shown are means ± SD for three independent measurements. Canton-S is the wild type, *Sply* indicates the homozygous *Sply*-null mutant, *lace* is the recessive lethal allele of serine palmitoyltransferase, $Sply^{14a}$ is the homozygous *Sply* revertant, and *Sk2* is the homozygous sphingosine kinase 2 null mutant.

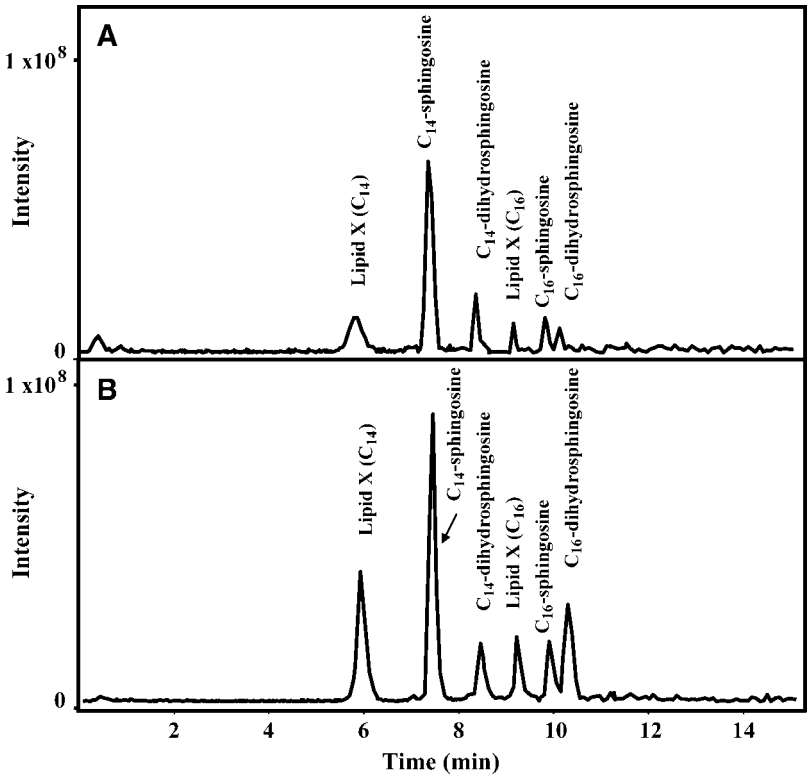

Fig. 1. LC-MS analysis of *Drosophila* lipid extracts after Strata C18-E column purification. Samples were separated on a C18 column with a gradient of methanol from 30 to 80% in 10 min, from 80 to 95% in 2 min, and at 95% for 3 min. After separation, the lipids were subjected to MS-ESI+ analysis and an extracted ion chromatogram was obtained for wild-type flies (A) and *Sply* flies (B). Peaks were identified as *m/z* 242.1 [lipid X (C_{14})], *m/z* 244.1 (C_{14}-sphingosine), *m/z* 246.1 (C_{14}-dihydrosphingosine), *m/z* 270.1 [lipid X (C_{16})], *m/z* 272.1 (C_{16}-sphingosine), and *m/z* 274.1 (C_{16}-dihydrosphingosine).

findings suggest that the enzymes in the sphingolipid pathway recognize and process molecules containing the $\Delta^{4,6}$-sphingadiene backbone and that these lipids likely have an endogenous function in *Drosophila*.

$\Delta^{4,6}$-Sphingadiene-containing sphingolipids in *Drosophila* development

To address the potential role of $\Delta^{4,6}$-sphingadiene-containing sphingolipids in development and *Drosophila* biology, we quantified these lipids in flies of various developmental stages. As shown in **Table 3**, $\Delta^{4,6}$-sphingadiene-containing sphingolipids were observed throughout development, with the exception of young embryos aged 0–12 h. This is in contrast to our previous findings for sphingosine- and dihydrosphingosine-containing sphingolipids, which were detectable in very young embryos aged 0–6 h and 6–12 h (18). After early embryogenesis and persistently throughout development and adulthood, *Sply* mutants demonstrated increased levels of $\Delta^{4,6}$-sphingadiene-containing sphingolipids compared with wild-type

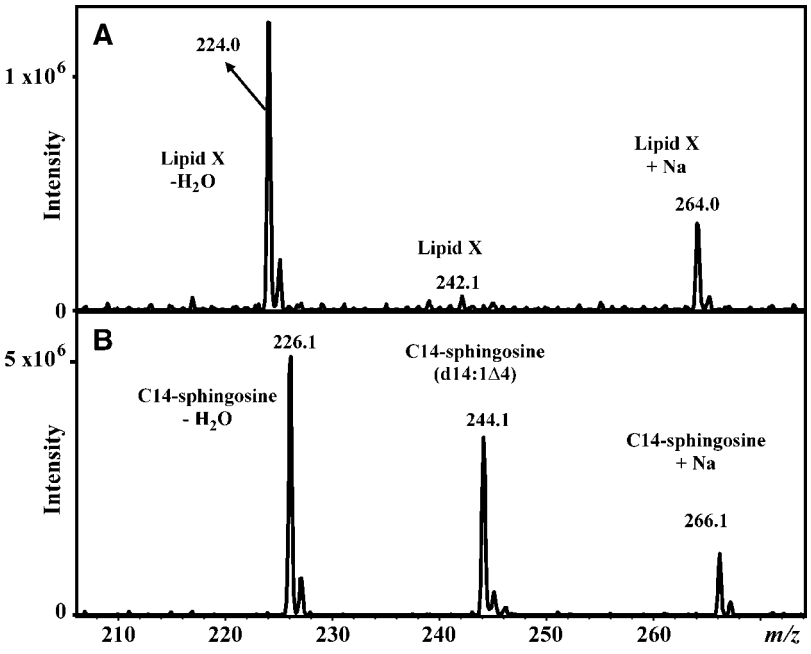

Fig. 2. LC-MS spectra of C_{14}-sphingosine and lipid X from *Drosophila*. Samples were separated as described in the legend to Fig. 1, and scans from *m/z* 200 to 280 were obtained from lipid X *m/z* 242.1 (A) and C_{14}-sphingosine *m/z* 244.1 (B).

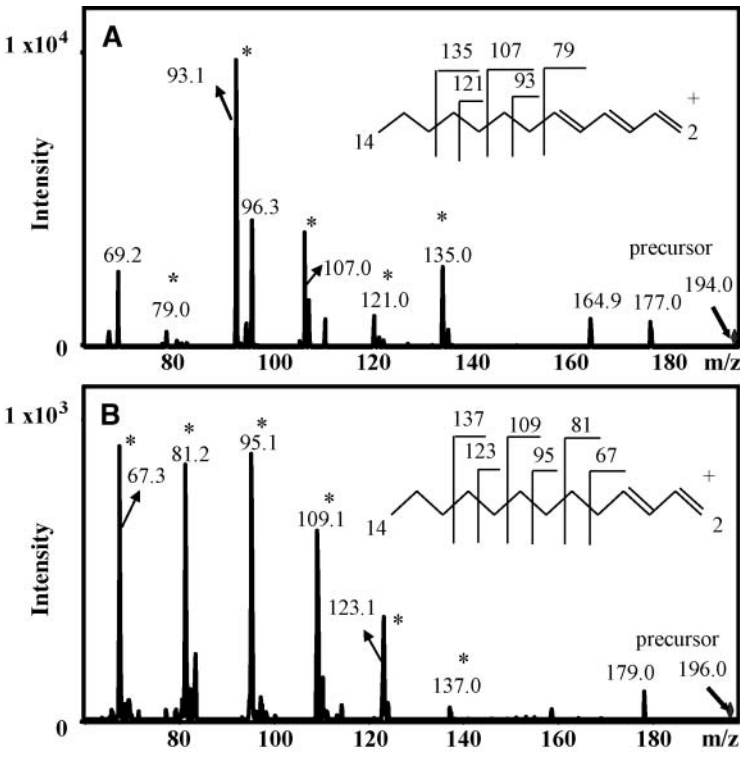

Fig. 3. LC-MS-ESI ion trap AutoMS^3 mass spectra of the water-loss component of C_{14}-sphingosine and lipid X from *Drosophila*. Samples were separated as described in the legend to Fig. 1, and scans from *m/z* 50 to 200 were obtained for the water-loss component of lipid X (*m/z* 224→194) (A) and the water-loss component of C_{14}-sphingosine (*m/z* 226→196) (B). Peaks originating from carbon chain fragmentation were identified and marked with asterisks.

flies. $\Delta^{4,6}$-Sphingadiene-containing LCBPs were not detectible in wild-type flies at any age, whereas in *Sply* mutants, $\Delta^{4,6}$-sphingadiene-containing LCBPs and ceramides accumulated beginning in late embryogenesis. This pattern is consistent with the observation that *Sply* gene transcription is low in early embryogenesis and peaks in the late embryonic stage at 12–24 h (15). Together, these findings suggest that the activity of SPLY is needed for proper regulation of the levels of $\Delta^{4,6}$-sphingadiene-containing sphingolipids. To test this directly, protein extracts from wild-type and *Sply* mutants were incubated with $\Delta^{4,6}$-C_{14}-sphingadiene for 1 h, followed by $\Delta^{4,6}$-C_{14}-sphingadiene quantification by HPLC. We found that wild-type but not *Sply* protein extracts were able to reduce $\Delta^{4,6}$-C_{14}-sphingadiene levels by almost 50% (data not shown).

Sply RNA interference in *Drosophila* S2 cells

To further verify a relationship between *Sply* and $\Delta^{4,6}$-sphingadiene-containing sphingolipids, RNAi was used to knock down SPLY expression in *Drosophila* S2 cells. Semiquantitative RT-PCR of *Sply* message in total RNA from RNAi-treated S2 cells 3 days after initiation of knockdown (day 3) demonstrated a 2.8 cycle delay compared with cells treated with a control RNAi. This indicates that specific RNAi reduced *Sply* gene expression by ~75%. Similarly, SPLY protein expression was reduced by ~70% on day 4, as determined by immunoblotting S2 cell extracts with antiserum generated against the C-terminal peptide of the SPLY protein (**Fig. 4**). The duration of SPLY knockdown was ~48–72 h, with the greatest effect at day 4. SPLY depletion was associated with a significant increase in both $\Delta^{4,6}$-sphingadiene LCBs and the corresponding $\Delta^{4,6}$-sphingadiene LCBPs. The level of $\Delta^{4,6}$-sphingadiene LCBs inversely correlated with SPLY levels throughout the time course (**Table 4**). These findings confirm that SPLY activity is required for the catabolism of $\Delta^{4,6}$-sphingadiene-containing sphingolipids.

Spatial accumulation of $\Delta^{4,6}$-sphingadiene-containing sphingolipids in *Drosophila* tissues

Although *Sply* gene expression in the developing embryo appears to be almost exclusively restricted to gut endoderm,

TABLE 3. LC-MS analysis of endogenous $\Delta^{4,6}$-sphingadiene-containing sphingolipids in different stages of development

Sphingolipid	Embryos, 0–6 h	Embryos, 6–12 h	Embryos, 12–18 h	Embryos, 18–24 h	Larvae First Instar	Larvae Third Instar	Adults
Wild type							
LCB	nd	nd	4.53 ± 0.87	13.24 ± 1.67	16.82 ± 2.11	14.21 ± 3.22	10.98 ± 3.77
LCBP	nd	nd	nd	nd	nd	nd	nd
Ceramide	nd	nd	74.55 ± 9.88	72.26 ± 5.33	81.43 ± 10.13	73.75 ± 6.44	98.76 ± 12.12
Sply^{05091}							
LCB	nd	nd	30.51 ± 4.89	46.65 ± 7.51	56.44 ± 5.29	62.27 ± 9.34	66.65 ± 13.45
LCBP	nd	nd	11.67 ± 1.89	12.33 ± 3.11	16.56 ± 2.45	21.16 ± 3.14	18.29 ± 2.67
Ceramide	nd	nd	124.54 ± 17.88	192.22 ± 12.73	130.78 ± 16.34	240.14 ± 23.23	221.32 ± 31.22

nd, not detectable. Values shown are means ± SD (pmol/mg protein) for three independent measurements.

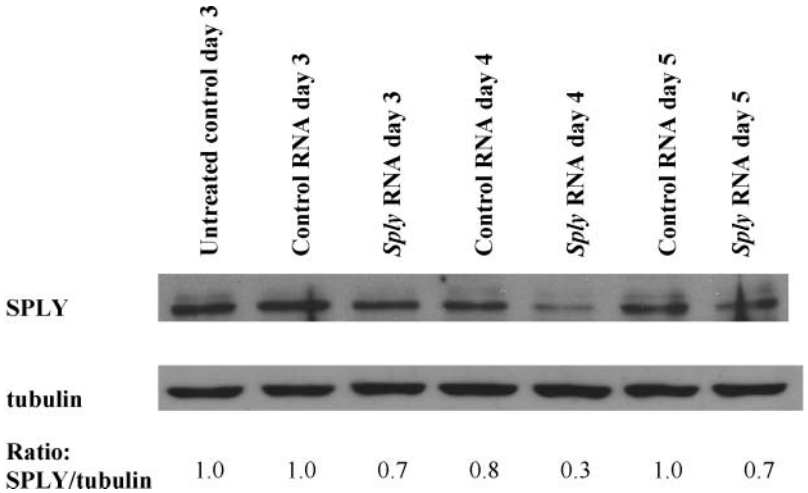

Fig. 4. Downregulation of SPLY by RNA interference in *Drosophila* S2 cells. *Drosophila* S2 cells were treated with double-stranded RNA against SPLY as described in Experimental Procedures. The efficiency of the knockdown was validated by Western blotting.

adult *Sply* mutants exhibit nonintestinal phenotypes, suggesting that *Sply* expression contributes to the global regulation of sphingolipids throughout the organism (15). Because LCBs, ceramides, and sphingadiene-ceramides have been implicated as mediators of apoptotic cell death, we considered the possibility that the accumulation of $\Delta^{4,6}$-sphingadienes and/or $\Delta^{4,6}$-sphingadiene-ceramides in specific tissues of the *Sply* mutant might contribute to their degeneration. Toward that end, wild-type and *Sply* adult flies were separated into thorax (which is largely composed of the dorsal longitudinal flight muscles), abdomen, head, and ovaries. As shown in **Table 5**, the highest levels of $\Delta^{4,6}$-sphingadienes and $\Delta^{4,6}$-sphingadiene-ceramides were found in the abdomen in both *Sply* and wild-type flies. However, the greatest differences between wild-type and mutant lines were observed in thoraces, where $\Delta^{4,6}$-sphingadienes were 28-fold higher, and in ovaries, where $\Delta^{4,6}$-sphingadienes were ~20-fold higher in *Sply* mutants than in wild-type controls. We have previously shown that adult *Sply* mutant flies exhibit increased levels of sphingolipids containing sphingosine or dihydrosphingosine LCBs (15). Therefore, we separated fly tissues and compared the levels of $\Delta^{4,6}$-sphingadienes and $\Delta^{4,6}$-sphingadiene-ceramides with the levels of the other major sphingolipid species. As shown in Table 5, there was an increase in total (sphingosine plus dihydrosphingosine) LCBs and ceramides in all tissues of the *Sply* mutant compared with the wild-type control. In the abdomen, head, and ovaries, we found similar increases in the levels of total LCBs, ceramides, $\Delta^{4,6}$-sphingadienes, and $\Delta^{4,6}$-sphingadiene-ceramides. However, in the thorax, we found a greater accumulation of the $\Delta^{4,6}$-sphingadiene compounds compared with the sphingosine and dihydrosphingosine compounds. This suggests that the flight muscle tissue of the adult fly is more prone to accumulate $\Delta^{4,6}$-sphingadienes in the absence of SPLY activity and that the $\Delta^{4,6}$-sphingadiene-containing compound likely play a role in the degeneration of this tissue.

$\Delta^{4,6}$-Sphingadiene-containing sphingolipids inhibit cell proliferation in *Drosophila* Cl.8 wing disc cells

To address whether endogenous $\Delta^{4,6}$-sphingadienes and ceramides containing $\Delta^{4,6}$-sphingadiene backbones affect cell proliferation in *Drosophila*, $\Delta^{4,6}$-C_{14}-sphingadiene and the corresponding C_2-sphingadiene-ceramide bearing a $\Delta^{4,6}$-C_{14}-sphingadiene LCB were synthesized and purified to homogeneity, as described in Experimental Procedures. The response to treatment with exogenous $\Delta^{4,6}$-sphingadiene-containing sphingolipids was evaluated in the *Drosophila* Cl.8 cell line. This line is derived from the larval imaginal wing disc, which gives rise to adult thoracic muscles as a result of a fusion event between wing disc myoblasts and larval muscle template fibers. Because of the lack of a true *Drosophila* myocyte line, this cell line was used as a representative of DLM cells. As shown in **Fig. 5**, incubation for 6 h with 20 μM $\Delta^{4,6}$-C_{14}-sphingadiene and the corresponding C_2-sphingadiene-ceramide diminished cell proliferation, as demonstrated by a decrease in the hydrolysis of the MTS compound. The effect from $\Delta^{4,6}$-C_{14}-sphingadiene and C_2-sphingadiene-ceramide on both cell proliferation and apoptosis was more pronounced than the effect from C_{14}-sphingosine and C_2-ceramide.

TABLE 4. HPLC analysis of $\Delta^{4,6}$-sphingadiene LCBs in *Drosophila* S2 cells treated with double-stranded RNA against SPLY

RNA	Day 3	Day 4	Day 5	Day 6	Day 7
SPLY RNA (pmol/mg protein)	12.05 ± 5.02	33.85 ± 4.14	56.02 ± 7.75	46.92 ± 3.38	22.21 ± 2.83
Control RNA (pmol/mg protein)	13.63 ± 3.64	10.66 ± 2.64	16.79 ± 3.92	12.45 ± 3.02	11.76 ± 4.82

Values shown are means ± SD for three independent measurements. Time points are shown as days after initiation of double-stranded RNA treatment.

TABLE 5. LC-MS analysis of endogenous $\Delta^{4,6}$-sphingadiene-containing sphingolipids in different tissues of 4–6 day old flies

Sphingolipid	Abdomen	Head	Ovaries	Thorax
Wild type				
LCB	17.90 ± 3.45	7.72 ± 1.22	3.07 ± 036	3.67 ± 0.99
Ceramide	145.00 ± 11.98	51.05 ± 4.66	61.10 ± 9.52	41.89 ± 3.33
Sply^{05091}				
LCB	172.31 ± 23.66 (9.6) [12.9]	72.73 ± 5.88 (9.4) [7.4]	87.73 ± 12.44 (28.6) [25.8]	72.19 ± 8.66 (19.7) [8.8]
Ceramide	618.35 ± 93.71 (4.3) [5.7]	231.07 ± 39.99 (4.5) [3.2]	158.28 ± 13.81 (2.6) [2.2]	263.59 ± 19.43 (6.3) [3.6]

Values shown are means ± SD (pmol/mg protein) for three independent measurements. Numbers in parentheses represent fold increase of $\Delta^{4,6}$-sphingadiene-containing sphingolipids compared with the wild type. Numbers in square brackets represent fold increase of sphingosine- and dihydrosphingosine-containing sphingolipids compared with the wild type.

DISCUSSION

In the current study, we used mass spectrometric approaches to characterize a family of endogenous diene-containing sphingolipids in *Drosophila*. The sphingadiene LCBs were either 14 or 16 carbons long and contained Δ-4,6 conjugated double bonds. The $\Delta^{4,6}$-sphingadiene LCB and corresponding LCBP and ceramide species require SPLY for their ultimate degradation, as shown by their marked accumulation in *Sply* mutant tissues. The most marked accumulation of $\Delta^{4,6}$-sphingadienes was observed in *Sply* thoracic muscles, which undergo tissue degeneration in the adult fly. Because genetic defects in sphingolipid synthesis abrogate this phenotype, we suspect that the $\Delta^{4,6}$-sphingadiene-containing sphingolipids contribute to tissue degeneration and that tight control over sphingolipid metabolism is necessary to maintain tissue integrity in the adult.

The structure of higher order sphingolipids has been studied in dipterans (23–27). However, until the last few years, relatively little information about sphingolipid structure and function in *Drosophila* has been available. One recent study defined the majority of higher order sphingolipids in *Drosophila* embryos (28). This study provides evidence that *Drosophila* sphingolipids are unique compared with other dipterans by the presence of 4-substituted rather than 3-substituted *N*-acetyl glycosamine as the penultimate residue in the N8 structure. Additionally, it was found that many sphingolipids of *Drosophila* contain phosphoethanolamine derivatization of *N*-acetyl glycosamine residues, a modification that potentially could induce significant structural alterations that influence glycolipid function. Compared with other dipterans, there are greater numbers of acidic glycolipids in *Drosophila*, all of which possess phosphoethanolamine groups, and the acidic structures possess longer chain lengths. These studies were performed entirely on embryos; therefore, developmental changes in glycosphingolipid structure or prevalence cannot be surmised. Studies of *Drosophila* glycosphingolipid mutants have revealed phenotypes that are affected in signaling pathways needed for embryonic development and oogenesis (29–31).

Sphingolipid metabolites have been studied extensively, and numerous reports have described the potent biological effects of LCBs, LCBPs, and ceramides (7–12). We previously characterized the sphingolipid metabolites in *Drosophila* and found that a disruption of their metabolic processing resulted in severe phenotypes, as observed in the *Sply* and *Sk2* mutants (14, 15, 17, 18). In this study,

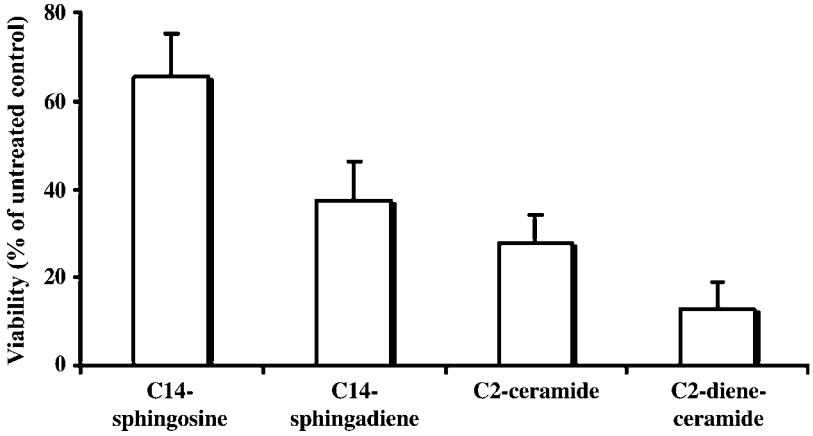

Fig. 5. Viability of *Drosophila* Cl.8 cell lines after treatment with $\Delta^{4,6}$-sphingadiene-containing sphingolipids. The *Drosophila* wing disk cell line Cl.8 was treated for 6 h with 20 μM long chain bases (LCBs) and 20 μM ceramide as described in Experimental Procedures. After treatment, cell viability was verified by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Values shown for viability are means ± SD for three independent measurements. A significant difference between treatment with lipids containing sphingosine and $\Delta^{4,6}$-sphingadiene was found for both LCBs and ceramides.

we identified a family of endogenous sphingolipids containing a $\Delta^{4,6}$-sphingadiene LCB. Although not studied extensively, $\Delta^{4,6}$-sphingadienes have been reported in insect species, indicating that these structures may be conserved among dipteran insects (19, 20). In contrast, sphingadiene LCBs, with the double bonds at C4 and C8, have been identified in numerous species. The $\Delta^{4,8}$-sphingadienes are commonly found in plants and plant-derived dietary constituents such as soy and legumes (32). In addition, they have also been detected as rare endogenous lipids in mammalian and human brain, aorta, and plasma (33–36). The $\Delta^{4,6}$-sphingadiene-containing sphingolipids accumulated markedly throughout development and in all tissues of the adult *Sply* mutant fly. Although we found an overall increase in the total amount of sphingolipid metabolites in the *Sply* mutant compared with the wild-type control, we found greater accumulation of the $\Delta^{4,6}$-sphingadiene-containing sphingolipids in the thorax and flight muscles. This finding suggests that a lack of tight regulation of these lipids may account for the flight muscle degeneration phenotype observed in the *Sply* mutant fly.

How SPLY activity regulates these lipids remains unknown. The mechanism responsible for the synthesis of $\Delta^{4,6}$-sphingadienes from precursor lipids is also unknown, although the introduction of a second double bond into the LCB by a sphingolipid desaturase or fatty acid desaturase is likely to be the route of synthesis. SPLY catalyzes the final step in sphingolipid degradation by cleaving a LCBP at the C2-C3 bond, forming a long chain aldehyde and phosphoethanolamine. The SPLY reaction is important for lipid homeostasis in *Drosophila*, as the phosphoethanolamine product is used for phosphatidylethanolamine biosynthesis and the regulation of sterol regulatory element binding protein processing (37). Therefore, a lack of SPLY activity results in an accumulation of sphingolipid metabolites and potentially an alteration of phospholipid metabolites and other indirect effects on lipid metabolism.

The future identification of the enzyme responsible for the introduction of the Δ-6 double bond in *Drosophila* and the generation of null mutants should elucidate the specific contribution of $\Delta^{4,6}$-sphingadienes to *Drosophila* development and tissue maintenance.

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