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Seminolipid and its precursor/degradative product, galactosylalkylacylglycerol, in the testis of saposin A- and prosaposin-deficient mice

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Abstract Sphingolipid activator proteins (saposins A, B, C, and D) are derived from a common precursor protein (prosaposin) and specifically activate in vivo degradation of glycolipids with short carbohydrate chains. A mouse model of prosaposin deficiency (prosaposin$^{-/-}$) closely mimics the human disease with an elevation of multiple glycolipids. The recently developed saposin A$^{-/-}$ mice showed a chronic form of globoid cell leukodystrophy, establishing the essential in vivo role of saposin A as an activator for galactosylceramidase to degrade galactosylceramide. Seminolipid, the principal glycolipid in spermatozoa, and its precursor/degradative product, galactosylalkylacylglycerol (GalEAG), were analyzed in the testis of the two mouse mutants by electrospray ionization mass spectrometry. Saposin A$^{-/-}$ mice showed the normal seminolipid level, while that of prosaposin$^{-/-}$ mice was ~150% of the normal level at the terminal stage. In contrast, GalEAG increased up to 10 times in saposin A$^{-/-}$ mice, whereas it decreased with age in the wild-type as well as in prosaposin$^{-/-}$ mice. These analytical findings on the two saposin mutants may shed some light on the physiological function of seminolipid and GalEAG.—Tadano-Aritomi, K., J. Matsuda, H. Fujimoto, K. Suzuki, and I. Ishizuka. **Seminolipid and its precursor/degradative product, galactosylalkylacylglycerol, in the testis of saposin A- and prosaposin-deficient mice.** ***J. Lipid Res.* 2003.** 44: **1737–1743.**

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Sphingolipid activator proteins (saposins A, B, C, and D) are small heat-stable glycoproteins, which are derived from a common precursor protein (prosaposin) (1). Prosaposin is encoded by a single gene and proteolytically processed to saposins A, B, C, and D. These four saposins are all homologous to each other, having six conserved cysteines and one common glycosylation site. In lysosomes, the saposins activate hydrolysis of glycolipids by lysosomal hydrolases. In spite of the structural similarities of the four saposins, their activator functions are specific, with some overlaps, for individual hydrolases. Human patients with mutations in the saposin B and C domains show phenotypes of metachromatic leukodystrophy and Gaucher disease, indicating that saposins B and C are essential for in vivo degradation of galactosylsulfatide [galactosylceramide (GalCer) I^3-sulfate] and glucosylceramide by arylsulfatase A and galactosylceramidase, respectively (2, 3). There are reports suggestive of saposin A being a galactosylceramidase activator (4, 5), and saposin D being a ceramidase activator (6, 7). However, it was unclear whether either saposin A or D is indispensable for normal cellular function. Recently, a mouse model was generated by introducing a mutation in the saposin A domain of the prosaposin gene (8). The clinical, biochemical, and pathological analyses of saposin A$^{-/-}$ mice indicated that saposin A is an indispensable activator of galactosylceramidase to degrade GalCer in vivo.

In addition to its well-documented role in lysosomal degradation of glycolipids, putative functions for secreted prosaposin have been proposed, including functions as a ganglioside binding/transport protein (9), a neurotrophic factor (10), and possible roles in gonadal development (11). The prosaposin gene may also code for the major sulfated glycoprotein of the Sertoli cell, which is postulated to be essential in the process of spermatogenesis (12, 13). Two mutations are known in human patients that result in complete lack of prosaposin and, consequently,

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all four processed saposins with a severe and complex phenotype (14, 15). An experimental mouse model of prosaposin deficiency (prosaposin$^{-/-}$) showed accumulation of multiple glycolipids in multiple organs, which closely mimicked the human disease (16).

Seminolipid [3-sulfogalactosyl-1-alkyl-2-acyl-*sn*-glycerol, galactosylalkylacylglycerol (GalEAG) I^3-sulfate] is the principal glycolipid in spermatozoa of mammals comprising, for example, approximately 3% of total lipids and more than 90% of total glycolipids in boar spermatozoa (17, 18). Seminolipid is synthesized by sulfation of its precursor, GalEAG, by the action of 3′-phosphoadenylylsulfate:cerebroside 3′-sulfotransferase (CST, EC 2.8.2.11) and is degraded to GalEAG by arylsulfatase A, the enzyme missing in metachromatic leukodystrophy (19). GalEAG is synthesized by galactosylation of alkylacylglycerol (EAG) by UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.62) and is likely to be degraded by galactosylceramidase, which is deficient in globoid cell leukodystrophy. CGT- or CST-deficient mice manifest complete inhibition of spermatogenesis before the first meiotic division, providing strong support for the view that seminolipid and/or GalEAG are indispensable for spermatogenesis (20, 21). Genetic abnormalities in the prosaposin gene may well also interfere with the metabolism and function of these galactolipids in the testis. We have analyzed seminolipid and GalEAG in the testis of the two mouse models, saposin A$^{-/-}$ and prosaposin$^{-/-}$ with a sensitive analytical method using mass spectrometry in order to investigate the effect of the saposin/prosaposin abnormalities on glycolipid metabolism and function of the testis.

MATERIALS AND METHODS

Mice

Saposin A$^{-/-}$ and prosaposin$^{-/-}$ mice were originally generated through gene targeting technology as previously described (8, 16). Affected mice (saposin A$^{-/-}$, prosaposin$^{-/-}$) were produced through carrier-to-carrier mating and the wild-type littermates from the saposin A$^{+/-}$ mating were used as controls. Mice were killed by decapitation, and the testes were quickly dissected and frozen at −80°C until lipid analysis. Simultaneously some testes were processed for the pathological evaluation. Prosaposin$^{-/-}$ mice had been backcrossed to the C57BL6/J strain, whereas saposin A$^{-/-}$ mice were the F2 generation and the genetic background was still a mixture of 129/SvJ and C57BL6/J. All animal protocols used in these studies have been approved by the Internal Review Board of the University of North Carolina and the University of Tokushima.

Histology

Testes were dissected and fixed in Bouin's solution overnight. After dehydration, tissues were embedded in paraffin, and 6 μm sections were stained for the periodic acid-Schiff reaction followed by hematoxylin staining.

Lipid extraction and analysis

Wild-type mice of 10, 16, 25, 76, and 109 days of age, saposin A$^{-/-}$ mice of 10, 15, 20, 28, 74, 118 and 141 days, and prosaposin$^{-/-}$ mice of 20 and 34 days were used. Testes obtained from two to six mice of each age and genotype were pooled and extracted with chloroform-methanol as described previously (20, 22). The total lipid extract was analyzed by two-dimensional TLC using the solvent systems, chloroform-methanol-water containing 0.2% $CaCl_2$ (65:25:4; v/v/v) for the first direction, and chloroform-methanol-3 M NH_4OH (65:25:4; v/v/v) or chloroform-methanol-acetone-acetic acid-water (8:2:4:2:1; v/v/v/v/v) for the second direction. The bands were visualized with orcinol (hexose-containing lipids), azure A (sulfolipids), or cupric acetate-phosphoric acid (phospholipids and other lipids), and determined by densitometry (CS-9000; Shimadzu, Kyoto, Japan) (20, 22).

Identification of lipids was performed by negative-ion liquid secondary ion mass spectrometry (LSIMS) on a Concept IH mass spectrometer (Shimadzu/Kratos, Kyoto, Japan) (23). Lipid bands on the TLC plate were transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-P; ATTO, Tokyo, Japan) by iron-blotting (Far-Eastern blotting) (24). The band on the membrane was excised and placed on a mass spectrometer probe tip with triethanolamine as the matrix (22, 25).

Partial purification and determination of seminolipid and GalEAG by electrospray ionization mass spectrometry combined with high-performance liquid chromatography

The total lipid extracts (∼2.3 mg) prepared from the testis (50 mg wet weight) of each group of mice were dissolved in 0.2 ml of chloroform and chromatographed on a prepacked silica gel column (Bond Elut SI 1 ml/100 mg; Varian, Harbor City, CA). After eluting nonpolar lipids (cholesterol, cholesterol ester, triacylglycerol, etc.) with 0.5 ml each of chloroform and chloroform-methanol (95:5; v/v), GalEAG and seminolipid were eluted with 0.5 ml each of chloroform-methanol, (90:10) and (85:15), respectively. The fractions were diluted with methanol (10–30 times for GalEAG and 50–80 times for seminolipid), and 5 μl portions were directly injected into a CAPCEL PAK C18 UG120 S-5 μm (1.5 × 35 mm) column (Shiseido, Tokyo) using the HP 1100 HPLC system (Agilent Technologies, Tokyo) (26) and eluted isocratically with methanol or methanol-water (9:1) for the determination of GalEAG or seminolipid, respectively, at 0.2 ml/min. Electrospray ionization mass spectrometry (ESIMS) was carried out on the LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) fitted with an ESI probe. A deactivated coated glass capillary, 0.1 mm ID, was used for the spray capillary. The heated capillary was set at 300°C, and the spray voltage set at 5.0 kV. The sheath gas and aux gas flow rates were set to 70 and 20 in an arbitrary unit, respectively. The tube lens offset was set at 0 V, which corresponds to the tube lens voltage of 120 V. Low-energy collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) (MS^2) was carried out using helium gas present in the ion trap. The collision gas pressure was 1.4×10^{-5} torr, and the relative collision energies used ranged from 40 to 45%. Concentrations of seminolipid and GalEAG in the samples were determined by ESIMS combined with high-performance liquid chromatography (HPLC) (LC-ESIMS) in the negative or positive ion mode, respectively. Selected reaction monitoring (SRM) was used as the detection mode, while the product ion at *m/z* 539 and 483, generated from the $[M - H]^-$ ion at *m/z* 795, and the $[M + Na]^+$ ion at *m/z* 739 were monitored for seminolipid or GalEAG, respectively (**Fig. 1**). The trap was run with automatic gain control, and two microscans were summed per scan.

RESULTS

Clinical phenotype and pathology of the testis

Saposin A$^{-/-}$ mice initially grew normally and were indistinguishable from their littermates. They developed slowly progressive hind leg paralysis with clinical onset at around

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Fig. 1. Electrospray ionization mass spectrometry (ESIMS) spectra of seminolipid and galactosylalkylacylglycerol (GalEAG). Seminolipid: negative-ion tandem mass spectrometry [MS/MS (MS^2)] spectrum of [M − H]$^-$ ion at m/z 795. GalEAG: positive-ion MS^2 spectrum of [M + Na]$^+$ ion at m/z 739.

2.5 months and survived up to 5 months (8). Both males and females were fertile, and they could produce several generations of pups beginning at around 60 days during their lifetime, indicating the nearly normal development of spermatogenesis in saposin A$^{-/-}$ mice. Histologically, no obvious abnormality was recognized in the testis.

Originally, 50% of prosaposin$^{-/-}$ mice died in utero, and those that were born were thought to consist of two distinct clinical phenotypes, i.e., neonatally fatal and later onset. The former (more than 50% of live births) might die within the first 24–48 h. The latter developed rapidly progressive severe neurological signs around 20 days and died by 35–38 days (16). As the mutant line was backcrossed to the C57BL6/J background, the proportion of live births steadily declined. The line can now be considered genetically homogeneous, and most prosaposin$^{-/-}$ mice die in utero and live births are rare. Only the testes from the latter group were analyzed. Although it was impossible to ascertain clinically if they were fertile, the testis weight of prosaposin$^{-/-}$ mice was not decreased even at the terminal stage of 34 days: the testis weights (mean) of prosaposin$^{-/-}$ (34 days, n = 2), saposin A$^{-/-}$ (28 days, n = 4), and wild-type mice (25 days, n = 3) were 51, 44, and 34 mg/testis, respectively. Histological examination showed no obvious evidence of abnormal spermatogenesis at 30 days, although the number of the late spermatids might be slightly reduced as compared with the normal littermates.

Determination of seminolipid and GalEAG by LC-ESIMS

ESIMS of seminolipid and GalEAG gave intense [M − H]$^-$ ion at m/z 795 and [M + Na]$^+$ ion at m/z 739, respectively. These ions are consistent with the EAG molecular species of 16:0 alcohol and 16:0 fatty acid. Other molecular species were observed at 28 Da higher, but their intensity was less than 5% of those at m/z 795 and 739. In the product ion spectra (MS^2) produced by CID of [M − H]$^-$ (m/z 795) or [M + Na]$^+$ (m/z 739) ions, elimination of the acyl residue was predominant, as demonstrated by the abundance of product ions at m/z 539 and 483, respectively (Fig. 1). Seminolipid and GalEAG in the samples, which were eluted as a single peak at about 2.5 min, were quantified by monitoring the ions at m/z 539 and 483, respectively, in the SRM mode. The calibration curves were linear from 0.25 to 3 nmol/ml, i.e., 0.5 to 6 pmol/5 μl injection, for both seminolipid and GalEAG with a correlation coefficient (R^2) of greater than 0.995. Because seminolipid is the major glycolipid in the testis, it could be determined using an aliquot of the total lipid extract without further purification. On the other hand, partial purification was a prerequisite for quantification of GalEAG, because it is a minor glycolipid, particularly in the testis of normal adult mice. For both seminolipid and GalEAG, complete purification is not necessary: the presence of other glycolipids and/or phospholipids did not interfere with the determination by LC-ESIMS^2 when large amounts of faster-eluting nonpolar lipids were removed by the prepacked silica gel column. To examine the recovery and the sample-to-sample variation of the method, the known amounts of seminolipid and GalEAG were added to the total lipid extract before passing through the column. As shown in **Table 1**, partial purification by the column did not reduce the recovery of seminolipid or GalEAG. The coefficients of variation of six separate experiments were less than 3% and those of the recoveries from four different samples, i.e., with or without addition of the three known amounts of seminolipid and GalEAG, were less than 9%. These results could confirm the reliability of the method. The precision of the method was further established by six separate determinations using purified glycolipids as well as the crude fractions containing seminolipid and/or GalEAG. The coefficients of variation were 0.4–1.6%. The detection limits for seminolipid and GalEAG were 10 and 50 pmol/ml, i.e., 0.02 and 0.1 pmol/injection, respectively, at a signal-to-noise ratio of 3.

GalEAG increases in saposin A$^{-/-}$ mice while seminolipid increases in prosaposin$^{-/-}$ mice

The putative bands on the TLC plates were identified as seminolipid and GalEAG with 16:0 alcohol and 16:0

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TABLE 1. Recovery of seminolipid and GalEAG

Added (A)		Determined (D)		Recovery (R)	
Seminolipid	GalEAG	Seminolipid	GalEAG	Seminolipid	GalEAG
		nmol		%	
0	0	40.7 ± 0.5[a]	11.6 ± 0.8		
1.8	0.9	42.5 ± 0.5	12.5 ± 1.0	103 ± 8[b]	105 ± 9[c]
5.4	2.7	46.1 ± 0.6	14.3 ± 1.4	99 ± 4	98 ± 6
16.2	8.1	56.6 ± 0.9	19.6 ± 2.1	98 ± 3	99 ± 5

GalEAG, galactosylalkylacylglycerol. The total lipid extract (~2.3 mg) prepared from the testis (50 mg wet tissue) of saposin $A^{-/-}$ mice (118 days of age), which contained 40.7 nmol of seminolipid and 11.6 nmol of GalEAG, was mixed with the known amount (A) of purified seminolipid and GalEAG. After passing through the prepacked silica gel column, an aliquot of the fractions containing seminolipid or GalEAG was determined by electrospray ionization mass spectrometry (ESIMS) combined with high-performance liquid chromatography (LC-ESIMS) as described in the text.

[a] Values are the mean ± coefficient of variation (%) of six separate experiments.

[b] Recovery (R) for seminolipid = [(D − 40.7)/A] × 100.

[c] R for GalEAG = [(D − 11.6)/A] × 100.

fatty acid by iron-blotting followed by negative-ion LSIMS (20). By visual examination of the two-dimensional TLC plates (**Fig. 2**), the band corresponding to GalEAG (1 in Fig. 2) was reduced in the testis of wild-type mice at 109 days and significantly increased in the testis of saposin $A^{-/-}$ mice at 141 days. The seminolipid band (2 in Fig. 2) was slightly increased in the testis of prosaposin$^{-/-}$ mice at 34 days.

Age-related changes in the level of GalEAG and seminolipid were determined by the LC-ESIMS[2] technique in the testis of saposin $A^{-/-}$ and prosaposin$^{-/-}$ mice in comparison with wild-type mice (**Fig. 3**). In the testis of wild-type mice, the level of GalEAG dramatically increased by 12 days, then reached maximum at around 16 days, and decreased rapidly to the adult level. A similar pattern was observed in the testis of prosaposin$^{-/-}$ mice. In the testis of saposin $A^{-/-}$ mice, by contrast, the GalEAG level kept increasing and reached 10 times as high as the normal level at around 70 days after birth (Fig. 3A).

In the testes of wild-type and saposin $A^{-/-}$ mice, the seminolipid level increased rapidly by 12 days concomitant with its precursor GalEAG, just before spermatocytes normally begin to appear at the zygotene stage (20, 27). The level of seminolipid then increased gradually by 17 days and remained almost constant until the terminal stage. In contrast, the testis of prosaposin$^{-/-}$ mice contained 150% of the normal level of seminolipid at their terminal stage of 34 days (Fig. 3B).

No significant changes in other lipids except sphingomyelin

TLC profiles of polar glycolipids, including longer chain neutral glycolipids and gangliosides, in the testis of saposin $A^{-/-}$ and prosaposin$^{-/-}$ mice were essentially similar as compared with age-matched littermates. No significant changes were observed in the concentration of major phospholipids, including phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and cardiolipin (data not shown). The level of sphingomyelin was ~170% of the normal level in the testis of prosaposin$^{-/-}$ mice at the terminal stage of 34 days (1.4 μmol/g wet tissue). The sphingomyelin levels in the testes of other groups of mice, including 20-day-old prosaposin$^{-/-}$ mice, were within the range of 0.7–0.9 μmol/g wet tissue.

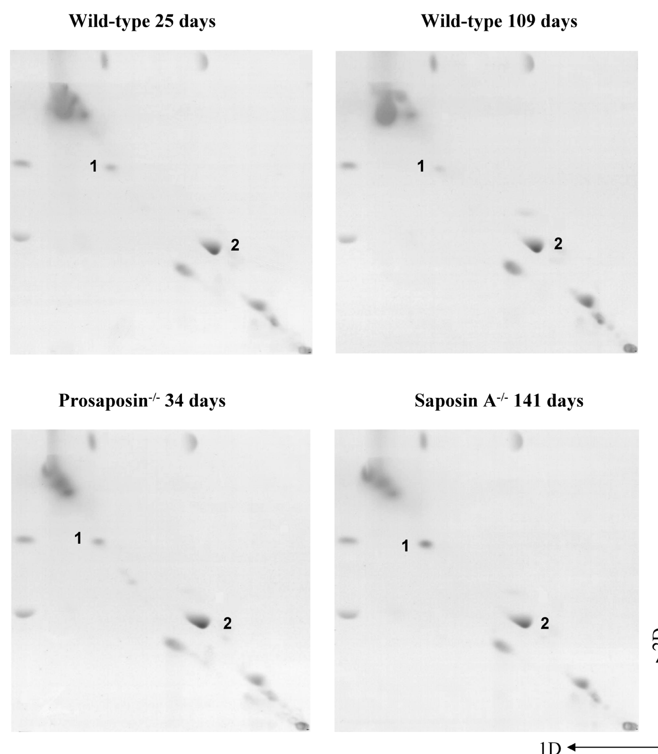

Fig. 2. Two-dimensional TLC of a total lipid extract from the testis of wild-type (25 and 109 days), prosaposin$^{-/-}$ mice (34 days), and saposin $A^{-/-}$ mice (141 days). An aliquot of the total lipid extract corresponding to 2.5 mg of testis was separated on a TLC plate with chloroform-methanol-water containing 0.2% $CaCl_2$ (65:25:4; v/v/v) for the first direction (1D), and chloroform-methanol-3 M NH_4OH (65:25:4; v/v/v) for the second direction (2D). Glycolipid bands were visualized with the orcinol reagent. Identification of components was performed by negative-ion liquid secondary ion mass spectrometry after iron-blotting: 1, GalEAG; 2, seminolipid. Seminolipid from boar testis (lower band) and GalEAG (upper band) prepared by desulfation of seminolipid (18) were applied as references in upper and left sides in each plate.

DISCUSSION

The gene encoding prosaposin is unusual in several respects (1). It encodes a precursor protein, prosaposin, which eventually is processed in the lysosome to the four homologous proteins, saposins A, B, C, and D. Existence of human patients deficient in either saposin B or C (2, 3) and an experimental mouse line deficient in saposin A (8) clearly indicates the relatively specific nature of the in vivo activating function of each of the saposins. On the other hand, prosaposin itself is postulated to have a few physiological functions, including as the Sertoli cell major sul-

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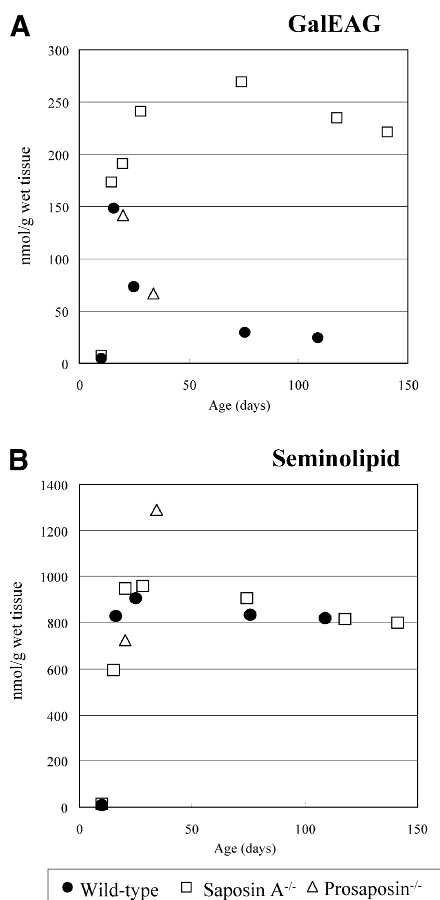

Fig. 3. Age-related changes of GalEAG and seminolipid levels in the testis of saposin $A^{-/-}$ and prosaposin$^{-/-}$ mice in comparison with wild-type mice. The fractions of individual glycolipids obtained from the prepacked silica gel column were determined by electrospray ionization tandem mass spectrometry combined with high-performance liquid chromatography as described in the text. Data are expressed as the means of duplicate experiments.

fated glycoprotein in the testis (12, 13). The precise physiological function of the Sertoli cell major sulfated glycoprotein is not known, but Morales et al. reported impaired spermatogenesis in a prosaposin-deficient mouse at 37 days (28). These previous findings clarified many aspects of the physiological functions of prosaposin and saposins. Nevertheless, many questions remain, particularly with respect to the function of the gene products in the testis. In this study, we report the results of our analytical studies on the testis of prosaposin$^{-/-}$ and saposin $A^{-/-}$ mice. Particular attention was focused on the characteristic lipid of the testis, seminolipid, and its metabolic precursor/degradative product, GalEAG. The former is degraded by arylsulfatase A, which is activated by saposin B and the latter by GalCer activated by saposin A.

New methodologies or modifications of existing methodologies needed to be developed first that would be suitable for analysis of the small amounts of glycolipids in mouse testis. A variety of procedures were examined for determining the sulfate ester content of various biomolecules, and some of them have been adopted for quantification of sulfoglycolipids including seminolipid. We recently adopted ion chromatography, that is, quantitation of ionic substances by ion conductimetry after separation by anion-exchange chromatography, for the determination of the sulfate liberated by hydrolysis of sulfolipids (29). It has the advantage of increased precision for wide concentration ranges and applicability to sulfolipids with various structures, because determination is based on liberated sulfate group using sodium or potassium sulfate as the standard. However, the sensitivity is not adequate for determination of minor components in the biological samples or cultured cells. The present results showed that the use of LC-ESIMS has a great advantage in sensitivity and can be applied not only to sulfoglycolipids but also to neutral glycolipids in the crude lipid extracts. By MS^2 in the SRM mode, the signal-to-noise ratio and the peak shape were greatly improved as compared with those on the selected ion monitoring chromatogram. Seminolipid as well as its nonsulfated counterpart, GalEAG, in the crude preparations could be determined specifically even in the presence of other lipids. Although the linear range for quantification is limited due to the characteristics of the ion trap analyzer, the detection limit was less than 0.1 pmol/injection, which is 1,000 times lower than that of the azure A method (30) or ion chromatography (29) for sulfated lipids.

By using the LC-ESIMS^2 technique, the age-related changes of seminolipid and GalEAG levels were determined in the testis of saposin $A^{-/-}$ and prosaposin$^{-/-}$ mice (Fig. 3). The GalEAG level increased up to 10 times the normal level in the testis of saposin $A^{-/-}$ mice, confirming that saposin A is indispensable for in vivo degradation of GalEAG as well as GalCer (8). Unexpectedly, the level of GalEAG decreased with age in the testis of prosaposin$^{-/-}$ mice, similar to that in the wild-type mice, despite the fact that the prosaposin$^{-/-}$ mouse is completely mRNA negative, i.e., no prosaposin, and consequently, none of the individual saposins, including saposin A, should be generated (16). Although we cannot offer an explanation for this paradox, the observation is firm and consistent with the much larger puzzle that prosaposin deficiency does not exhibit any of the phenotype of globoid cell leukodystrophy (i.e., saposin A deficiency) despite the absence of saposin A in the prosaposin$^{-/-}$ mouse (8, 16).

In spite of the increased level of the precursor GalEAG, the level of seminolipid was normal in the testis of saposin $A^{-/-}$ mice at all ages tested, suggesting that the activity of CST, which sulfates GalEAG to seminolipid, is regulated to maintain the seminolipid level within the normal range. In contrast, the seminolipid level in the testis of prosaposin$^{-/-}$ mice at 34 days was 150% of the normal level (Fig. 3B). The result could suggest that saposin B activates in vivo degradation of seminolipid as well as galactosylsulfatide for several reasons: *1*) While we could present only two data points, these derived from pooled samples and represent averages of the pooled testis. Given the precision of our analytical methodology, this ensures correct overall estimates, although it conversely obscures biological varia-

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tions among individual testes. *2*) The level of seminolipid at 34 days is twice as high as that at 20 days in the testis of prosaposin$^{-/-}$ mice (Fig. 3B). This may suggest the accumulation of seminolipid with age, although we could not follow the age-related changes because of the exceedingly rare live births and limited life span (35–38 days) of prosaposin$^{-/-}$ mice. *3*) It is well established that saposin B is an activator for arylsulfatase A, which is deficient in metachromatic leukodystrophy, and that seminolipid is a substrate of arylsulfatase A (19). *4*) The seminolipid level increases in the tissue of arylsulfatase A$^{-/-}$ mice (Molander-Melin, Månsson, Fredman, et al., personal communication). While these points provide only circumstantial support, they are collectively consistent with saposin B deficiency being the cause of seminolipid accumulation in the testis of the prosaposin$^{-/-}$ mouse.

These results indicated that testicular germ cells at the leptotene-zygotene stage, when biosynthesis of GalEAG and seminolipid begins, are not affected by lack of saposins or prosaposin. Normal spermatogenesis in saposin A$^{-/-}$ mice would suggest that either *1*) accumulation of GalEAG, which should be restricted to lysosomes, may not affect the process of spermatogenesis, or *2*) when the seminolipid level is kept within the normal range, spermatogenesis is normal regardless of the GalEAG level, although the arrested stage of spermatogenesis in CST-deficient mice is somewhat later than that in CGT-deficient mice (21).

Morales et al. (28) reported that 37-day-old prosaposin$^{-/-}$ mice showed several abnormalities in their reproductive organs, including testis, prostate, seminal vesicles, and epididymis. They showed that smaller testes (30% reduction in weight) of prosaposin$^{-/-}$ mice are associated with reduced spermiogenesis, the late spermatids being particularly affected. In the present study, however, histological analyses of the testis of saposin A$^{-/-}$ mice showed that the mutation at the saposin A domain in the prosaposin molecule does not affect the process of spermatogenesis. Furthermore, we did not observe any morphological defects in the testis or obvious abnormality in spermiogenesis, at least at 30 days of age in the prosaposin$^{-/-}$ mice lacking prosaposin itself. Although the reason for the apparent discrepancy between our findings and those of Morales et al. (28) is not clear, our results would pose the question of whether prosaposin, as the major sulfated glycoprotein of the Sertoli cell, is indispensable for normal spermatogenesis. We have not been able to test the possibility that spermatogenesis in prosaposin$^{-/-}$ mice is normal at 30 days but reduced at 37 days, because of the exceedingly rare live births of homozygous prosaposin$^{-/-}$ mice ever since the genetic background of the line was made homogeneous to the C57BL6/J strain. Even when homozygous prosaposin$^{-/-}$ mice are born, very few survive to 37 days. Recently, a spermatogonial cell line, which can differentiate into spermatocytes and spermatids in the presence of stem cell factor, was created (31). It was reported that sperm tails were not evident, although round spermatids were generated in the absence of Sertoli cells. These results may suggest that prosaposin is indispensable only for the final phase of spermatogenesis, which consists of a morphological transformation of spermatids. Insofar as the first wave of spermatogenesis is complete at 34 days in mice, it might not be ruled out that spermatogenesis in prosaposin$^{-/-}$ mice is reduced at the final phase, which could cause abnormalities in the testis at 37 days, as described by Morales et al. (28).

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REFERENCES

1. Sandhoff, K., T. Kolter, and K. Harzer. 2001. Sphingolipid activator proteins. *In* The Metabolic and Molecular Basis of Inherited Disease. C. R. Sciver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 3371–3388.
2. Christomanou, H., A. Aignesberger, and R. P. Linke. 1986. Immunochemical characterization of two activator proteins stimulating enzymic sphingomyelin degradation in vitro. Absence of one of them in a human Gaucher disease variant. *Biol. Chem. Hoppe Seyler.* **367:** 879–890.
3. Stevens, R. L., A. L. Fluharty, H. Kihara, M. M. Kaback, L. J. Shapiro, B. Marsh, K. Sandhoff, and G. Fischer. 1981. Cerebroside sulfatase activator deficiency induced metachromatic leukodystrophy. *Am. J. Hum. Genet.* **33:** 900–906.
4. Morimoto, S., B. M. Martin, Y. Yamamoto, K. A. Kretz, J. S. O'Brien, and Y. Kishimoto. 1989. Saposin A: second cerebrosidase activator protein. *Proc. Natl. Acad. Sci. USA.* **86:** 3389–3393.
5. Harzer, K., B. C. Paton, H. Christomanou, M. Chatelut, T. Levade, M. Hiraiwa, and J. S. O'Brien. 1997. Saposins (sap) A and C activate the degradation of galactosylceramide in living cells. *FEBS Lett.* **417:** 270–274.
6. Klein, A., M. Henseler, C. Klein, K. Suzuki, K. Harzer, and K. Sandhoff. 1994. Sphingolipid activator protein D (sap-D) stimulates the lysosomal degradation of ceramide in vivo. *Biochem. Biophys. Res. Commun.* **200:** 1440–1448.
7. Azuma, N., J. S. O'Brien, H. W. Moser, and Y. Kishimoto. 1994. Stimulation of acid ceramidase activity by saposin D. *Arch. Biochem. Biophys.* **311:** 354–357.
8. Matsuda, J., M. T. Vanier, Y. Saito, J. Tohyama, K. Suzuki, and K. Suzuki. 2001. A mutation in the saposin A domain of the sphingolipid activator protein (prosaposin) gene results in a late-onset, chronic form of globoid cell leukodystrophy in the mouse. *Hum. Mol. Genet.* **10:** 1191–1199.
9. Hiraiwa, M., S. Soeda, Y. Kishimoto, and J. S. O'Brien. 1992. Binding and transport of gangliosides by prosaposin. *Proc. Natl. Acad. Sci. USA.* **89:** 11254–11258.
10. O'Brien, J. S., G. S. Carson, H. C. Seo, M. Hiraiwa, and Y. Kishimoto. 1994. Identification of prosaposin as a neurotrophic factor. *Proc. Natl. Acad. Sci. USA.* **91:** 9593–9596.
11. Sprecher-Levy, H., A. Orr-Urtreger, P. Lonai, and M. Horowitz. 1993. Murine prosaposin: expression in the reproductive system of a gene implicated in human genetic diseases. *Cell Mol. Biol.* **39:** 287–299.
12. Collard, M. W., S. R. Sylvester, J. K. Tsuruta, and M. D. Griswold. 1988. Biosynthesis and molecular cloning of sulfated glycoprotein 1 secreted by rat Sertoli cells: sequence similarity with the 70-kilodalton precursor to sulfatide/GM1 activator. *Biochemistry.* **27:** 4557–4564.
13. Sylvester, S. R., C. Morales, R. Oko, and M. D. Griswold. 1989. Sulfated glycoprotein-1 (saposin precursor) in the reproductive tract of the male rat. *Biol. Reprod.* **41:** 941–948.
14. Schnabel, D., M. Schröder, W. Fürst, A. Klein, R. Hurwitz, T. Zenk, J. Weber, K. Harzer, B. C. Paton, A. Poulos, K. Suzuki, and K. Sandhoff. 1992. Simultaneous deficiency of sphingolipid activator pro-

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teins 1 and 2 is caused by a mutation in the initiation codon of their common gene. *J. Biol. Chem.* **267:** 3312–3315.
15. Hulková, H., M. Cervenková, J. Ledvinová, M. Tochácková, M. Hrebícek, H. Poupetová, A. Befekadu, L. Berná, B. C. Paton, K. Harzer, A. Böör, F. Smíd, and M. Elleder. 2001. A novel mutation in the coding region of the prosaposin gene leads to a complete deficiency of prosaposin and saposins, and is associated with a complex sphingolipidosis dominated by lactosylceramide accumulation. *Hum. Mol. Genet.* **10:** 927–940.
16. Fujita, N., K. Suzuki, M. T. Vanier, B. Popko, N. Maeda, A. Klein, M. Henseler, K. Sandhoff, H. Nakayasu, and K. Suzuki. 1996. Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids. *Hum. Mol. Genet.* **5:** 711–725.
17. Ishizuka, I., M. Suzuki, and T. Yamakawa. 1973. Isolation and characterization of a novel sulfoglycolipid, 'seminolipid,' from boar testis and spermatozoa. *J. Biochem. (Tokyo).* **73:** 77–87.
18. Ishizuka, I. 1997. Chemistry and functional distribution of sulfoglycolipids. *Prog. Lipid Res.* **36:** 245–319.
19. Yamaguchi, S., K. Aoki, S. Handa, and T. Yamakawa. 1975. Deficiency of seminolipid sulphatase activity in brain tissue of metachromatic leucodystrophy. *J. Neurochem.* **24:** 1087–1089.
20. Fujimoto, H., K. Tadano-Aritomi, A. Tokumasu, K. Ito, T. Hikita, K. Suzuki, and I. Ishizuka. 2000. Requirement of seminolipid in spermatogenesis revealed by UDP-galactose:ceramide galactosyltransferase-deficient mice. *J. Biol. Chem.* **275:** 22623–22626.
21. Honke, K., Y. Hirahara, J. Dupree, K. Suzuki, B. Popko, K. Fukushima, J. Fukushima, T. Nagasawa, N. Yoshida, Y. Wada, and N. Taniguchi. 2002. Paranodal junction formation and spermatogenesis require sulfoglycolipids. *Proc. Natl. Acad. Sci. USA.* **99:** 4227–4232.
22. Tadano-Aritomi, K., T. Hikita, H. Fujimoto, K. Suzuki, K. Motegi, and I. Ishizuka. 2000. Kidney lipids in galactosylceramide synthase-deficient mice. Absence of galactosylsulfatide and compensatory increase in more polar sulfoglycolipids. *J. Lipid Res.* **41:** 1237–1243.
23. Tadano-Aritomi, K., H. Kubo, P. Ireland, M. Okuda, T. Kasama, S. Handa, and I. Ishizuka. 1995. Structural analysis of mono- and bis-sulfated glycosphingolipids by negative liquid secondary ion mass spectrometry with high- and low-energy collision-induced dissociation. *Carbohydr. Res.* **273:** 41–52.
24. Taki, T., and D. Ishikawa. 1997. TLC blotting: application to microscale analysis of lipids and as a new approach to lipid-protein interaction. *Anal. Biochem.* **251:** 135–143.
25. Hikita, T., K. Tadano-Aritomi, N. Iida-Tanaka, J. K. Anand, I. Ishizuka, and S. Hakomori. 2001. A novel plasmal conjugate to glycerol and psychosine ("glyceroplasmalopsychosine"): isolation and characterization from bovine brain white matter. *J. Biol. Chem.* **276:** 23084–23091.
26. Hikita, T., K. Tadano-Aritomi, N. Iida-Tanaka, H. Toyoda, A. Suzuki, T. Toida, T. Imanari, T. Abe, Y. Yanagawa, and I. Ishizuka. 2000. Determination of N-acetyl- and N-glycolylneuraminic acids in gangliosides by combination of neuraminidase hydrolysis and fluorometric high-performance liquid chromatography using a GM3 derivative as an internal standard. *Anal. Biochem.* **281:** 193–201.
27. Bellvé, A. R., J. C. Cavicchia, C. F. Millette, D. A. O'Brien, Y. M. Bhatnagar, and M. Dym. 1977. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J. Cell Biol.* **74:** 68–85.
28. Morales, C. R., Q. Zhao, S. Lefrancois, and D. Ham. 2000. Role of prosaposin in the male reproductive system: effect of prosaposin inactivation on the testis, epididymis, prostate, and seminal vesicles. *Arch. Androl.* **44:** 173–186.
29. Tadano-Aritomi, K., T. Hikita, A. Suzuki, H. Toyoda, T. Toida, T. Imanari, and I. Ishizuka. 2001. Determination of lipid-bound sulfate by ion chromatography and its application to quantification of sulfolipids from kidneys of various mammalian species. *J. Lipid Res.* **42:** 1604–1608.
30. Tadano-Aritomi, K., and I. Ishizuka. 1983. Determination of peracetylated sulfoglycolipids using the azure A method. *J. Lipid Res.* **24:** 1368–1375.
31. Feng, L-X., Y. Chen, L. Dettin, R. A. Pera, J. C. Herr, E. Goldberg, and M. Dym. 2002. Generation and in vitro differentiation of a spermatogonial cell line. *Science.* **297:** 392–395.

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