

# Quantification of seminolipid by LC-ESI-MS/MS-multiple reaction monitoring: compensatory levels in *Cgt*<sup>+/-</sup> mice<sup>§</sup>

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**Abstract** Seminolipid, also known as sulfogalactosylglycerolipid (SGG), plays important roles in male reproduction. Therefore, an accurate and sensitive method for SGG quantification in testes and sperm is needed. Here we compare SGG quantitation by the traditional colorimetric Azure A assay with LC-ESI-MS/MS using multiple reaction monitoring (MRM). Inclusion of deuterated SGG as the internal standard endowed accuracy to the MRM method. The results showed reasonable agreement between the two procedures for purified samples, but for crude lipid extracts, the colorimetric assay significantly overestimated the SGG content. Using ESI-MS/MS MRM, C16:0-alkyl/C16:0-acyl SGG of *Cgt*<sup>+/-</sup> mice was quantified to be 406.06 ± 23.63 μg/g testis and 0.13 ± 0.02 μg/million sperm, corresponding to 78% and 87% of the wild-type values, respectively. CGT (ceramide galactosyltransferase) is a critical enzyme in the SGG biosynthesis pathway. *Cgt*<sup>-/-</sup> males depleted of SGG are infertile due to spermatogenesis arrest. However, *Cgt*<sup>+/-</sup> males sire offspring. The higher than 50% expression level of SGG in *Cgt*<sup>+/-</sup> animals, compared with the wild-type expression, might be partly due to compensatory translation of the active CGT enzyme. The results also indicated that 78% of SGG levels in *Cgt*<sup>+/-</sup> mice were sufficient for normal spermatogenesis.—Kongmanas, K., H. Xu, A. Yaghoubian, L. Franchini, L. Panza, F. Ronchetti, K. Faull, and N. Tanphaichitr. **Quantification of seminolipid by LC-ESI-MS/MS-multiple reaction monitoring: compensatory levels in *Cgt*<sup>+/-</sup> mice.** *J. Lipid Res.* 2010. 51: 3548–3558.

**Supplementary key words** liquid chromatography • electrospray ionization • tandem mass spectrometry

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Seminolipid, 1-*O*-alkyl-2-*O*-acyl[β-D-(3'-sulfoxy)galactopyranosyl(1'-3)]*sn*-glycerol, also known as sulfogalactosylglycerolipid (SGG) and SM4g (Fig. 1A), is present selectively and substantially in mammalian male germ cells, comprising about 10 mol% of total lipids (1). SGG consists of glycerol with a *sn*-1 alkyl and *sn*-2 acyl chain as the lipid backbone, and a (3'-sulfo)-galactopyranose β-linked to the *sn*-3 position. Consistently across mammalian species studied so far, C16:0/C16:0 SGG is the main molecular species (1, 2), although minor molecular species, such as C16:0/C14:0, C14:0/C16:0, C15:0/C16:0, C17:0/C16:0, C16:0/C18:0, C18:0/C16:0, and C17:0/C18:0 SGG, representing < 10% of the main species, have also been described (3, 4). Accumulated evidence has pointed to the significance of SGG for mammalian male reproduction (1). SGG on the sperm surface is involved in sperm-zona pellucida (ZP) binding and sperm-egg plasma membrane binding (5, 6). As a structurally ordered lipid, SGG participates in the formation of lipid rafts in the sperm head, which act as platforms for ZP binding (7–10). Genetic deletion of *Cgt* and *Cst*, coding for two enzymes (ceramide galactosyltransferase and cerebroside sulfotransferase, respectively) in the SGG biosynthetic pathway, leads to spermatogenesis arrest in the primary spermatocyte stage and, thus, male infertility (11, 12).

Due to its physiological significance, an accurate, sensitive, specific method for SGG quantification is needed. A simple colorimetric Azure A assay has been conventionally

Abbreviations: CGT, ceramide galactosyltransferase; HPTLC, high-performance thin-layer chromatography; LOD, limit of detection; MRM, multiple reaction monitoring; PNS, post-nuclear supernatant; SGC, sulfogalactosylceramide; SGG, sulfogalactosylglycerolipid.

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used (13, 14). Azure A is a cationic dye that reacts with anionic lipids, including sulfolipids (15, 16). Azure A is also useful for staining sulfolipids on thin layer chromatography (TLC) plates. The limit of detection (LOD) of the colorimetric assay for sulfolipids is around 10  $\mu\text{g}$  in the original method and, despite modifications and improvements, is still around 0.3  $\mu\text{g}$  (13, 17). The LOD of Azure A staining of chromatographed lipids on high-performance (HP)TLC plates is even higher: at least 1  $\mu\text{g}$  of a sulfolipid is required for detection. While the Azure A assay is convenient and simple, it has drawbacks, including lack of sensitivity and specificity, as it reacts with all sulfolipids and certain anionic lipids, such as cardiolipin (15, 16). HPTLC can be used to separate sulfolipids from other lipid classes; therefore, HPTLC coupled with Azure A staining remains a useful procedure for obtaining a global picture of sulfolipids in a lipid extract. More precise quantification of SGG in a lipid extract can be made by scraping the SGG band from the HPTLC plate. SGG extracted from the silica powder can then react with Azure A in solution. However, SGG molecular species that differ by subtle changes in the acyl/alkyl chains are usually not resolved from one another by HPTLC. Therefore, individual minor SGG molecular species in a sample would be overlooked by the HPTLC-Azure A-based method; further, the amount of the major C16:0/C16:0 isoform would be overestimated by Azure A reaction, if the collective amount of the minor SGG molecular species is significant. A method precisely targeted to specific SGG molecular species is required for accurate quantification.

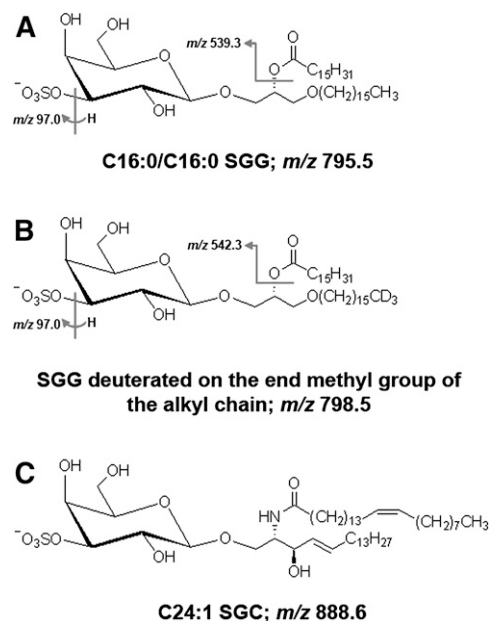
Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) with multiple reaction monitoring would be an ideal quantification method for SGG molecular species. It has been used for quantification of sulfogalactosylceramide (SGC) (also known as sulfatide and SM4s), a sulfolipid analog of SGG (18). Both SGG and SGC carry the same galactosyl sulfate head group, but unlike SGG with a glycerol backbone, SGC has a sphingosine skeleton (Fig. 1C). SGC is the better studied sulfolipid, as its accumulation in the brain and other tissues due to a genetically inherited deficiency of arylsulfatase A (ASA) or saposin B (SGC carrier protein) results in metachromatic leukodystrophy, a fatal neurological disease (19, 20). With a suitable internal standard, LC-MS/MS-MRM quantification can be supremely accurate and sensitive, as it can quantify individual sulfolipid isoforms that differ by relatively subtle structural changes in the hydrocarbon chains (18, 21–23). The best internal standards are stable isotope-labeled analogs ( $^2\text{H}$ -,  $^{13}\text{C}$ -, and  $^{15}\text{N}$ -labeled forms) because their physical and chemical properties, including extraction efficiency, chromatographic behavior, and ionization efficiency, are virtually identical to those of the natural, nonlabeled analyte. Although LC-MS/MS-MRM analysis has recently been used to determine the amounts of seminolipid in saposin A- and prosaposin-deficient mice (23), the analyses were performed without an internal standard and relied on absolute signal intensity uncorrected for sample losses during extraction. In this study, we compared SGG quantification on the same samples using the colorimetric assay and the LC-MS/MS-MRM procedure

with specific measurement of the major C16:0/C16:0 isoform using  $^2\text{H}_3$ -C16:0-alkyl/C16:0-acyl SGG, synthesized as previously described (24) as the internal standard. Further, the MRM method was used to quantify C16:0/C16:0 SGG in testes and sperm of fertile *Cgt* heterozygous male mice. The results allowed a correlation between the testicular and sperm SGG levels and the fertility status of *Cgt* $^{+/-}$  mice. This information is relevant to future studies, including a determination of the minimal level of SGG required to maintain male fertility.

## MATERIALS AND METHODS

### Materials

Silica gel HPTLC plates (60  $\text{\AA}$ , 200  $\mu\text{m}$  thickness, 10  $\times$  10 cm) were purchased from Whatman (Kent, UK). Acetonitrile (Optima<sup>®</sup> grade) was from Fisher Scientific (Pittsburgh, PA). Chloroform and methanol (HPLC grade) were from EMD chemicals, purchased through VWR (Mississauga, ON, Canada). Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) were from Doosan Serdary Research Laboratories (Eaglewood Cliffs, NJ). Cholesterol (Chol) and SGC were from Sigma-Aldrich (St. Louis, MO). SGG,  $^2\text{H}_3$ -SGG, and galactosylglycerolipid (GG) were prepared in-house as described below. Azure A and Coomassie Brilliant Blue G250 were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ultrapure water (16–18  $\text{m}\Omega\cdot\text{cm}^{-1}$ ) was produced by an ELGA water purification system (Wycombe, UK). All other reagents and solvents were of analytical grade or better. Rabbit polyclonal IgG antibody directed against a CGT peptide (RNGKYKGNRVRKHEKKVR: residues 524–541) was generated by AnaSpec Inc. (San Jose, CA).



**Fig. 1.** A: Structure of C16:0/C16:0 SGG (1-*O*-hexadecyl-2-*O*-hexadecanoyl-3-*O*- $\beta$ -D-(3'-sulfo)-galactopyranosyl-*sn*-glycerol). B: Deuterium-labeled internal standard. C: C24:1 sulfatide (also known as cerebroside sulfate and sulfogalactosylceramide, SGC), the other main mammalian sulfolipid. MS/MS fragmentation positions that generate ions used for MRM analyses are indicated. SGG, sulfogalactosylglycerolipid.

## Animals

CD-1 male and female mice (4- to 6-weeks-old) were purchased from Charles River Canada (St-Constant, QC, Canada). One wild-type male and two *Cgt*<sup>+/-</sup> female mice (both having C57BL/6J genetic background), previously generated by Dr. Brian Popko (formerly at the University of North Carolina at Chapel Hill, Chapel Hill, North Carolina) (25), were used to start the colony that was maintained by continuing breeding of heterozygous males with heterozygous females. DNA isolated from tail tissues was used for genotyping by PCR following the previously described protocol (26) with two sets of primers for DNA amplification: *Cgt* (forward), 5'-CTC TCA GAA GGC AGA GAC ATT GCC-3' and *Cgt* (reverse), 5'-CAT CCA TAG GCT GGA CCC ATG AAC-3' (this set gave a wild-type gene product of ~500 bp); *Neo* (forward), 5'-GGA GAG GCA ATT CGG CTA TGA C-3' and *Neo* (reverse), 5'-CGC ATT GCA TCA GCC ATG ATG G-3' (this set gave a ~300 bp product of the inserted neomycin gene in the exon 2 of the *Cgt* sequence in *Cgt*<sup>-/-</sup> mice) (25). All mice were kept in a temperature-controlled room (22°C) with a 12:12 dark/light cycle. The boarding and handling of these mice, as well as all subsequent experimental procedures with them, were approved by Animal Care Committee, Ottawa Hospital Research Institute (OHRI).

## Mouse testis and sperm preparation

Mice were sacrificed by cervical dislocation. Testes were removed, decapsulated, weighed (~100–120 mg/testis) and individually hand-homogenized in 1 ml of phosphate buffered saline (PBS), which was then used for lipid extraction. Sperm were retrieved from the caudal epididymis and vas deferens from each animal into 1 ml of HEPES-buffered Krebs Ringer solution (KRB-HEPES; 119.4 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM Mg<sub>2</sub>SO<sub>4</sub>, 4 mM NaHCO<sub>3</sub>, 1 mM sodium pyruvate, 25 mM sodium lactate, 5.6 mM glucose, 1 U/ml of penicillin G, 1 µg/ml of streptomycin sulfate, 21 mM HEPES, and 28 µM phenol red at pH 7.4) (14). For lipid extraction, the sperm suspension was centrifuged (430 g, 10 min, 25°C), then pellet was washed twice with PBS and finally resuspended in 0.5 ml PBS. For the in vitro fertilization experiments, the sperm suspension was subjected to Percoll gradient centrifugation to select for the motile population as previously described (27). Sperm numbers were counted under a microscope in a 10 µl aliquot of the sperm suspension using a hemocytometer.

## Lipid extraction from mouse testes and sperm

Lipids were extracted from testes of a CD-1 male, and three sets of *Cgt*<sup>+/-</sup> males and their wild-type littermates, as well as from caudal epididymal and vas deferens sperm retrieved from the individual *Cgt* heterozygous and wild-type mice. Following a modified Bligh-Dyer method (14, 28–30), methanol (2.5 ml) and chloroform (1.25 ml) was added to the 1 ml PBS testis homogenate. After thorough mixing and 1 h incubation at room temperature, the sample was diluted further with chloroform and water (1.25 ml each), then vigorously mixed and centrifuged (800 g, 5 min, room temperature). The lower phase was transferred to a clean glass tube, and the upper phase was reextracted twice with chloroform (2.5 ml each time), again using centrifugation to separate the phases. The lower phases containing extracted total lipids from two testes of the same animal were then pooled and dried in a stream of nitrogen for storage at -20°C until further use. Lipids were also extracted from the sperm suspension from 1 animal (18–25 million sperm) using the same procedure as described for the testes.

Testis lipids from the CD-1 male were divided into two portions. Partially purified SGG was prepared from one portion by preparative HPTLC (see below). SGG quantification by Azure A

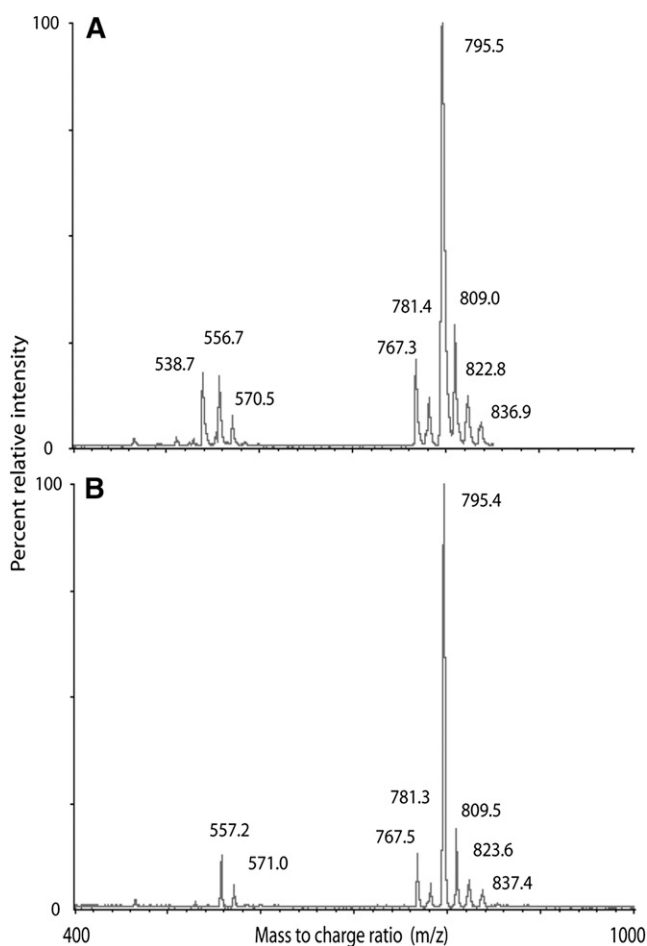
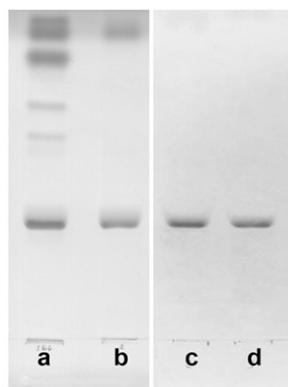
assay and ESI-MS/MS-MRM analyses, as well as analytical HPTLC, was performed on CD-1 testis lipids (with and without further purification for SGG), and *Cgt*<sup>+/-</sup> and *Cgt*<sup>+/+</sup> total testis and sperm lipids.

## Preparation of SGG and GG

**SGG from pig testis.** Partially purified SGG starting material was previously prepared in bulk amount from pig testis total lipid extracts via Biosil-A column chromatography followed by preparative TLC (29, 30). It contained a prominent band with an *R<sub>f</sub>* at 0.33 and other components when examined by HPTLC with Coomassie Blue staining (Fig. 2, top panel, lane a). This material, referred to as “partially purified SGG,” was stored as powder at -20°C. Then 30 µg was dissolved in chloroform/methanol (1/1, v/v) and divided into two equal fractions, one of which was further purified by preparative HPTLC (29). HPTLC plates were predeveloped in chloroform/methanol (1/1, v/v), air dried, and heat activated at 100°C for 1 h prior to use. Lipids were then loaded as a broad band (8 cm), with SGG standard (highly purified from pig testis in our lab and characterized by ESI-MS/MS (24)) loaded as a separate narrow band (0.8 cm) on the side. The plates were developed with chloroform/methanol/water (65/25/4, v/v/v), air dried, and placed in a closed jar of iodine vapor, which transiently stained the lipids yellow. The band corresponding to SGG (based on the *R<sub>f</sub>* value, and the same position as the cochromatographed SGG standard) was scraped and extracted three times with chloroform/methanol (1/1, v/v). The pooled extracts were dried in a stream of nitrogen and then subjected to Bligh-Dyer partitioning. This final product, referred to as “highly purified SGG,” appeared as a single band with an *R<sub>f</sub>* of 0.33, as visualized by analytical HPTLC/Coomassie Blue staining (see methodology below) (Fig. 2, top panel, lane b). Both the partially purified and highly purified SGG samples revealed similarity of the *m/z* 97 parent ion ESI tandem mass spectra (Fig. 2, bottom panel), and both possessed the expected major signal at *m/z* 795.4 corresponding to the C16:0/C16:0 SGG anion (calculated 795.52982 Da for C<sub>41</sub>H<sub>79</sub>O<sub>12</sub>S). However, both samples still contained other copurified sulfated components (Fig. 2, bottom panel), highlighting the difficulty of preparing a molecularly homogeneous standard from natural sources. Therefore, the highly purified SGG sample was still inadequate for use as a quantitative standard for MRM experiments, although it could still be used as a standard in analytical HPTLC. Both the partially purified (the unprocessed 15 µg fraction) and the highly purified SGG samples were used for the initial comparison of the two SGG quantification methods—the Azure A colorimetric assay and LC-MS/MS MRM analysis (Table 1).

**SGG from CD-1 mouse testes.** Half of the total lipids extracted from both testes of the CD-1 male mouse was used to prepare partially purified mouse testis SGG following the same preparative HPTLC method as described for the pig testis SGG. Total mouse testis lipids and partially purified SGG samples were subjected to both quantification methods (Table 1), as well as analytical HPTLC and parent (sulfate) ion ESI tandem mass spectrometry.

**Galactosylalkylacylglycerol.** GG was prepared from partially purified pig testis SGG via mild acid hydrolysis (31). Following HPTLC with Coomassie Blue staining, the product showed a single band with an *R<sub>f</sub>* of 0.659. During positive ion ESI in the presence of lithium, the final product gave an intense ion at *m/z* 723.3 (24) corresponding to the lithiated adduct (calculated as 723.5953 for C<sub>41</sub>H<sub>80</sub>O<sub>9</sub>Li). This sample was quantified by weighing and used as the HPTLC reference standard.



**Fig. 2.** Purity of pig testis SGG standards. Top panel: HPTLC of partially purified SGG (lanes a and c) and highly purified SGG (lanes b and d). Plates were treated with Coomassie Blue (lanes a and b) and Azure A (lanes c and d). The samples were prepared from a pig testis as described in "Materials and Methods." Bottom panel: Sulfate ( $m/z$  97) parent ion electrospray tandem spectra of the same partially purified SGG (A) and highly purified SGG (B) samples.

$^2\text{H}_3$ -SGG. Deuterated C16:0/C16:0 SGG with  $\text{C}^2\text{H}_3$  as the terminal methyl group in the alkyl chain (Fig. 1B) was chemically synthesized as previously described (24). The final product was molecularly homogeneous by NMR and mass spectral criteria. Its sodium salt form was quantified by weighing and used as the internal standard and quantitative reference for LC-MS/MS-MRM experiments.

## High-performance thin-layer chromatography

Conditions for analytical HPTLC plate preparation and development were the same as described for preparative HPTLC. Lipids dissolved in chloroform/methanol (20  $\mu\text{l}$ , 1/1, v/v) were loaded as a 0.8 cm band onto the preactivated HPTLC plate. Samples loaded included (A) partially purified pig testis SGG (3.75  $\mu\text{g}$ ); (B) highly purified pig testis SGG [3.75  $\mu\text{g}$  prepared from a parallel sample of (A)]; (C) total mouse testis lipids extracted from 5 mg of tissue; and (D) partially purified mouse testis SGG prepared from a parallel sample of (C). After plate development, lipids were detected by various dyes. These included Coomassie Brilliant Blue G-250 (0.03% in 30% methanol/100 mM NaCl at room temperature), the excess of which was removed with 30% methanol in 100 mM NaCl (14, 32); and Azure A (0.016% Azure in 1 mM  $\text{H}_2\text{SO}_4$ ), the excess of which was removed with 50 mM  $\text{H}_2\text{SO}_4$ /methanol (3/1, v/v) (7, 14). Coomassie Blue stained all lipids blue, whereas Azure A stained sulfolipids blue (29). Lipid standards run on each plate included SGG, GG, PE, PC, Chol, and SM. Digital images were recorded after staining using an Epson scanner (Epson Canada, ON, Canada).

## SGG quantification

*Azure A assay.* The method described by Kean (15) and as modified by Weerachayanukul et al. (13) was used. Dried lipid extracts were redissolved in chloroform/methanol (1/1, v/v), and aliquots corresponding to 7 million sperm or 1 mg wet weight of testes were transferred to screw-capped glass tubes and dried under  $\text{N}_2$ .  $\text{CHCl}_3/\text{MeOH}$  (1.5 ml, 1/1, v/v), aqueous  $\text{H}_2\text{SO}_4$  (1.5 ml, 50 mM), and Azure A dye solution (0.12 mg in 0.3 ml of 2.5 mM  $\text{H}_2\text{SO}_4$ ) were added to each tube. After thorough mixing and centrifugation (300 g, 10 min, 4°C), the chloroform phase containing the blue Azure A-lipid complex was transferred to a new glass tube, and the absorbance at 635 nm was recorded (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, Piscataway, NJ). Sperm samples were assayed in duplicate, and testis samples were assayed in triplicate. Sulfated lipid concentrations were inferred from interpolation from a standard curve constructed with SGC (0–10  $\mu\text{g}$ ) as standard. The LOD of this assay was about 0.3  $\mu\text{g}$  of SGC.

*LC-ESI-MS/MS-MRM.* Dried lipid samples (from ~200  $\mu\text{g}$  of testes or 1.4 million sperm) were redissolved in acetonitrile/methanol/water/acetic acid (41/23/36/1, v/v/v/v, 180  $\mu\text{l}$ ) to which was added  $^2\text{H}_3$ -SGG internal standard (100 pmoles in 20  $\mu\text{l}$  of chloroform/methanol, 1/1, v/v). Aliquots of this solution were diluted as necessary and injected (typically 100  $\mu\text{l}$ ) onto a reverse-phase HPLC column (C18, 150  $\times$  2.1 mm, Supelco Ascentis® Express) equilibrated in buffer A (methanol/water, 95/5, v/v, containing 1 mM ammonium acetate) and eluted (100  $\mu\text{l}/\text{min}$ ) with an increasing concentration of buffer B (chloroform/water, 500/0.2, v/v, containing 1 mM ammonium acetate; min/%B: 0/0, 5/0, 55/100). The effluent from the column was passed to an Ionspray® source connected to a triple-quadrupole mass spectrometer (Sciex API III+ Perkin Elmer-Sciex Instruments, Thornhill, ON, Canada) in which both Q1 and Q3 were tuned to unit resolution with about 10% valley between the  $^{13}\text{C}$ -isoforms of the polypropylene glycol (PPG) calibrant ions. Data was collected in the negative ion MS/MS-MRM mode using instrument manufacturer-supplied software (RAD version 2.6-FPU) with transitions selected for C16:0/C16:0 SGG ( $m/z$  795.5 $\rightarrow$ 97.0 and 795.5 $\rightarrow$ 539.3) and  $^2\text{H}_3$ -C16:0/C16:0 SGG (798.5 $\rightarrow$ 97.0 and 798.5 $\rightarrow$ 542.3) under previously optimized conditions [orifice –120 volts, collision gas (argon) thickness at an instrumental setting of 200, 1.8 s per scan, dwell time 250 msec per transition]. Peak areas and heights for the SGG and  $^2\text{H}_3$ -SGG transitions were

TABLE 1. Comparison of sulfolipid and C16:0/C16:0 SGG amounts measured by the colorimetric Azure A assay and by LC-MS/MS-MRM analysis

Sample	Sulfolipids		C16:0/C16:0 SGG	Fold Difference
	Azure A Assay	LC-ESI-MS/MS		
Pig testis SGG samples	$\mu\text{g}$		$\mu\text{g}$	
1. Partially purified pig testis SGG <sup>a</sup>	13.6 ± 1.6		9.6 ± 0.8	1.4
2. Highly purified pig testis SGG <sup>a</sup>	10.4 ± 2.4		8.4 ± 0.8	1.2
Mouse testis samples	$\mu\text{g/g testis}$		$\mu\text{g/g testis}$	
3. Total mouse testis lipid extract <sup>b</sup>	3816.9 ± 112.8		523.4 ± 38.1	7.3
4. Partially purified mouse testis SGG <sup>b</sup>	2003.6 ± 92.9		436.6 ± 56.5	4.6

SGG, sulfogalactosylglycerolipid.

<sup>a</sup>Thirty micrograms of partially purified pig testis SGG was weighed and divided into two equal fractions, one of which was used to prepare the highly purified sample, as described in "Materials and Methods" (see Fig. 2 for HPTLC patterns and sulfate ion ESI-MS/MS spectra). All assays were done in triplicate. The SGG amounts in the original fraction (15  $\mu\text{g}$  by weight) were reported as mean ± SD of the triplicate results.

<sup>b</sup>SGG quantification by both methods was done in triplicate on total mouse testis lipids and SGG partially purified by preparative HPTLC from the total testis lipids, as described in "Materials and Methods" (see Fig. 3 for HPTLC patterns and sulfate ion ESI-MS/MS spectra). The amounts of lipids used for each SGG quantification method were in the same ranges as those described for pig testis SGG. Data were expressed as mean ± SD of SGG per g of testis wet weight from the triplicate results.

recorded using instrument manufacturer-supplied software (MacSpec version 3.3). Calculations of the amount of C16:0/C16:0 SGG in each sample were made from the amount of internal standard added to each sample adjusted by the relative intensity of the two sets of signals, assuming 100% purity of the internal standard synthesized as the sodium salt. The average of the data obtained from the two transitions was used in the calculations. All lipid samples were analyzed in triplicate. The LOD of the MRM analysis was about 1 pmole of SGG.

### Sulfate ion scanning of testis lipids

Dried lipid samples were redissolved and injected into the same LC/MS/MS system as described above, with the mass spectrometer operating in the negative ion parent scan mode with the same source and MS/MS conditions. In these experiments, Q1 was tuned to unit resolution (10% valley between the PPG <sup>13</sup>C-isoforms), Q3 was tuned to lower resolution (no separation between the PPG <sup>13</sup>C isoforms), Q1 was scanned ( $m/z$  400–1500, 0.3 Da step size, 6 s/scan), and Q3 was set to transmit the  $m/z$  97.1 ion corresponding to HSO<sub>4</sub><sup>-</sup>.

### Fertility assessment of *Cgt*<sup>+/-</sup> male mice

**Mating experiments.** Five pairs of *Cgt*<sup>+/-</sup> males and their wild-type male littermates from five different litters (two pairs of three-month-old and three pairs of nine-month-old males) were used. Each male was individually caged with an eight-week-old wild-type female in the evening. The females were checked for vaginal plugs the following morning. If positive, this was counted as day 1 of gestation. As females of this strain are infanticidal, the pregnant females were euthanized between day 14 and 18 of gestation, prior to the expected delivery date of the pups. An accurate census of litter sizes was achieved by counting the number of fetuses or implantation sites in the uteri.

**In vitro fertilization.** To compare fertilizing ability of *Cgt*<sup>+/-</sup> sperm with that of the wild-type, in vitro fertilization (IVF) was carried out as previously described (33, 34). Four pairs of *Cgt*<sup>+/-</sup> males (two pairs of three-month-old and two pairs of nine-month-old males) and their wild-type male littermates were used. Mature eggs were collected from superovulated CD-1 females. Fertilization rate was defined as percentage of total eggs fertilized in each sample. A minimum of 80 eggs were used for the assessment of sperm fertilizing ability for each genotype.

### RNA purification and analysis

Total RNA was extracted from *Cgt*<sup>+/-</sup> and wild-type testes using an RNeasy kit (Qiagen, Mississauga, ON, Canada). The *Cgt*<sup>-/-</sup> samples were used as a negative control. The RNA was first quantified by A260 nm, and the same amount of RNA from each sample was used for reverse transcription using a Retroscript Kit (Ambion, Foster City, CA). The cDNA products were purified from other components in the reactions using a QIAquick PCR Purification Kit (Qiagen). To prepare *Cgt* cDNA for the quantitative real-time PCR, an aliquot of the cDNA products from reverse transcription of the wild-type sample was further subjected to PCR using the specific primer sets for *Cgt*, forward: 5'-CTC TCA GAA GGC AGA GAC ATT GCC-3' and reverse: 5'-CAT CCA TAG GCT GGA CCC ATG AAC-3'. The amplified *Cgt* PCR product was purified as described above and subjected to agarose gel electrophoresis and ethidium bromide staining (26). The stained *Cgt* PCR product was quantified by densitometric analyses by comparison to the intensity of DNA markers using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The cDNA samples of each mouse genotype (*Cgt*<sup>+/+</sup>, *Cgt*<sup>+/-</sup>, and *Cgt*<sup>-/-</sup>) and different dilutions of purified *Cgt* cDNA were used as templates in 20  $\mu\text{l}$  fluorometric quantitative PCR reactions. Each reaction mixture included 1 $\times$  Quantitect SYBR Green PCR master mix (Qiagen) and 0.5  $\mu\text{M}$  of the primer set. The PCR was performed in a Roche LightCycler version 2.0 (Roche Diagnostics, Laval, Quebec, Canada). The PCR was started with 15 min initial activation of Tag polymerase at 95°C, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 54°C for 30 s, and extension at 72°C for 40 s. The software for Roche LightCycler version 2.0 was used to construct a standard curve of cycle threshold (CT) values from all dilutions of *Cgt* cDNA. The concentration of the PCR product (mRNA) from each genotype sample was then determined from this standard curve based on its CT value. The concentration of *Cgt*<sup>+/-</sup> mRNA was expressed as a relative value to that of the *Cgt*<sup>+/+</sup> mRNA.

### Immunoblotting of CGT

Testes collected from *Cgt*<sup>+/-</sup> and wild-type littermates were decapsulated and individually homogenized in 1 ml of homogenization buffer (250 mM sucrose containing 1 mM EDTA and 10 mM Hepes, pH 7.2). Post-nuclear supernatants (PNS) were obtained by centrifugation (375 g, 15 min) (35). Aliquots of PNS were quantified for total proteins using the Bio-Rad Bradford assay with various concentrations of BSA to build a standard curve.

Equivalent amounts of protein (20 µg) from each PNS sample were subjected to SDS-PAGE (10% polyacrylamide, 1.0 mm thick) (36), followed by electroblotting onto a nitrocellulose membrane (37). The nitrocellulose was blocked for 1 h with 5% fat-free milk in Tris-buffered saline (137 mM NaCl in 20 mM Tris-HCl, pH 7.6) containing 0.05% Tween 20. Immunoblotting was performed using anti-CGT antiserum (1:5,000 dilution) as the primary antibody, and goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (1:5,000 dilution, Bio-Rad Laboratories, Hercules, CA) as the secondary antibody. The primary and secondary antibodies were prepared in the blocking medium. The reactivity of antibody-antigen was detected by enhanced chemiluminescence (ECL) using an ECL kit (Pierce, Rockford, IL). Relative CGT expression levels in the *Cgt<sup>+/−</sup>* samples compared with those in the wild-type samples were determined by densitometric analysis using AlphaImager Software (AlphaEase version 5.5, Alpha Innotech, San Leandro, CA).

### CGT enzymatic activity

The post-nuclear supernatants (prepared as described above) of the *Cgt<sup>+/−</sup>*, wild-type, and knockout mouse testes were used for the CGT activity assay, following a previously described protocol with some modifications (35). The assay was based on the ability of CGT to transfer galactose to C6-NBD-ceramide (N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]D-erythro-sphingosine) to form NBD galactosylceramide (NBD-GC), a fluorescent product. The post-nuclear supernatant (20 µg of protein) of each sample was incubated (25 min, 37°C) in a 200 µl reaction mixture containing homogenization buffer (described above) with 1% BSA, 2 mM UDP-galactose, 2 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, and 50 µM C6-NBD-ceramide (Avanti Polar Lipids, Alabaster, AL), after which chloroform (250 µl) and methanol (500 µl) were added. After mixing vigorously, additional chloroform and water (250 µl of each) was added. The samples were mixed again before centrifugation (800 g, 5 min, room temperature). The lower chloroform phase was transferred to another glass tube and dried under a stream of nitrogen. The residue was resolubilized in 20 µl of chloroform and applied to a HPTLC plate on which standard C6-NBD-GC (Avanti Polar Lipids) was also loaded. The plate was developed using the same solvent system as described in the HPTLC section, and the chromatographed fluorescent lipids were detected by a Typhoon 8600 Scanner (Molecular Dynamics). Densitometric analysis of NBD-GC in the samples was done using AlphaImager software.

### Statistical analyses

Student's *t*-test was used to analyze significant differences between two sets of data from the *Cgt<sup>+/−</sup>* and wild-type samples. A *P* value of less than 0.05 was considered statistically significant.

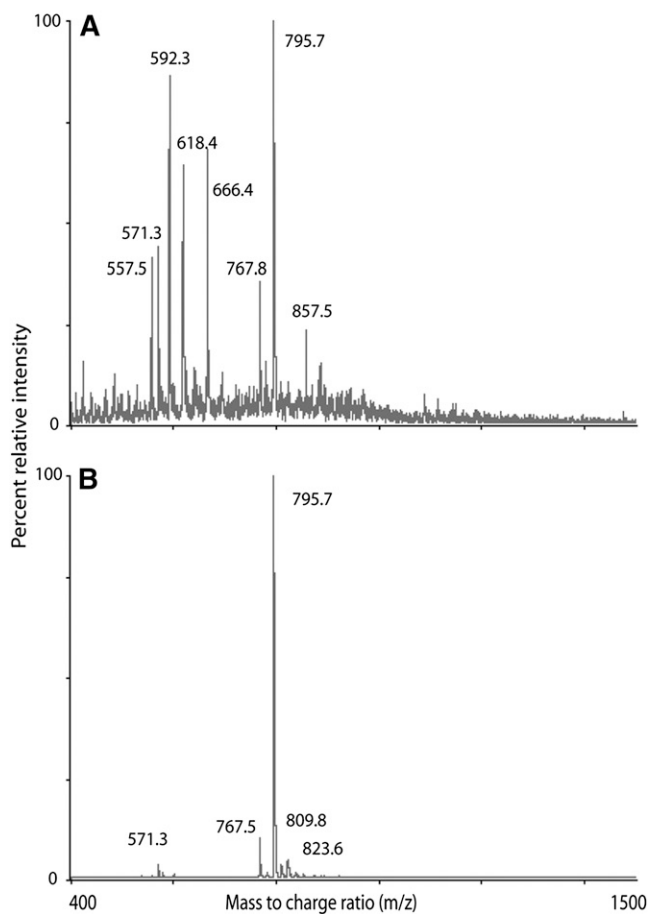
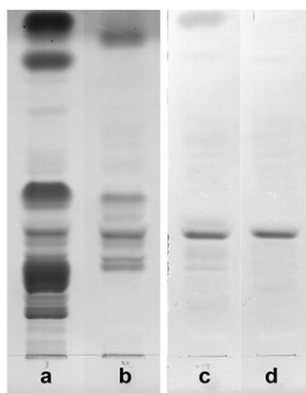
## RESULTS

### Quantification of SGG by Azure A and LC-ESI-MS/MS-MRM

Azure A quantitation consistently overestimated the SGG content of the biological samples compared with LC-ESI-MS/MS-MRM, which was targeted for only C16:0/C16:0 SGG. However, the magnitude of the overestimation was variable and depended on the purity of the lipid preparations, with high overestimation in crude lipid extracts (Table 1). Both partially and highly purified pig testis SGG samples were separated from major lipid classes via Biosil-A column chromatography. Therefore, relatively

small overestimation by the colorimetric procedure (1.2- and 1.4-fold for highly purified and partially purified pig testis samples, respectively) was observed (Table 1). This was likely the result of the presence of minor SGG isoforms and possibly trace levels of other Azure A-positive components in the samples. ESI-MS/MS parent ion (sulfate) scanning of partially and highly purified pig testis samples gave similar spectra, with C16:0/C16:0 SGG being the main molecular species (*m/z* 795) detected (Fig. 2, bottom panel). The presence of a single Azure A-stained SGG band on the HPTLC plate corroborated these MS results (Fig. 2, top panel, lanes c and d). Some of the other small MS signals in the *m/z* 750–850 range were likely to be minor SGG isoforms: C17:0/C18:0 for *m/z* 837; C16:0/C18:0 for *m/z* 823; C17:0/C16:0 for *m/z* 809; C15:0/C16:0 for *m/z* 781, and C14:0/C16:0 or C16:0/C14:0 for *m/z* 767 (3, 4). There were a number of putative sulfate-containing ions in the *m/z* range 450–600 in the pig testis SGG samples that were reduced in abundance but not completely removed by the additional purification to produce the highly purified sample (Fig. 2, bottom panel, A versus B). While the *m/z* 557 ion matched the lyso form of C16:0/C16:0 SGG, the identities of other signals in this *m/z* range were unknown. The decrease of these low-range molecular mass sulfolipids in the highly purified pig testis SGG sample would partly account for the lower sulfolipid amounts obtained from the Azure A assay compared with the partially purified SGG sample (Table 1, 24% decrease). However, this decrease could also come from the removal of other Azure A-reactive anionic lipids during the purification process. A significant reduction of Coomassie Blue-stained lipid bands on the HPTLC plate supported this statement (Fig. 2, top panel, lane a versus b). Nonetheless, this reduction was partly due to the lipid loss during the purification. The amount of C16:0/C16:0 SGG determined by LC-MS/MS-MRM was decreased by 13% (9.6 versus 8.4 µg, Table 1) before and after preparative HPTLC purification, indicating the lipid loss during purification.

In the mouse testis lipid samples, the sulfolipid content, as quantified by the Azure A procedure, was much higher than the amount of C16:0/C16:0 SGG obtained from LC-MS/MS-MRM (i.e., 7.3- and 4.6-fold difference for the total lipid extract and partially purified samples, respectively) (Table 1). HPTLC Coomassie Blue results revealed, as expected, a complex profile for the total lipid extract and a less complex pattern for the partially purified sample (Fig. 3, top panel, lanes a and b). While the HPTLC Azure A profile for both samples was dominated by the SGG band, additional but faint bands of low *R<sub>f</sub>* values were evident, especially in the total lipid extract (Fig. 3, top panel, lanes c and d). ESI-MS/MS parent ion (sulfate) scanning of total mouse testis lipids gave a complex spectrum dominated by C16:0/C16:0 SGG (Fig. 3, bottom panel, A). Therefore, the presence of substantial amounts of other sulfolipids in the total mouse testis lipid extract was partly the cause of a great difference (7.3-fold) in the levels of total sulfolipids over C16:0/C16:0 SGG. However, ESI-MS/MS parent ion (sulfate) scanning spectrum was relatively clean for the partially purified mouse testis lipid, with the C16:0/C16:0 SGG signal at *m/z* 795 accounting for more



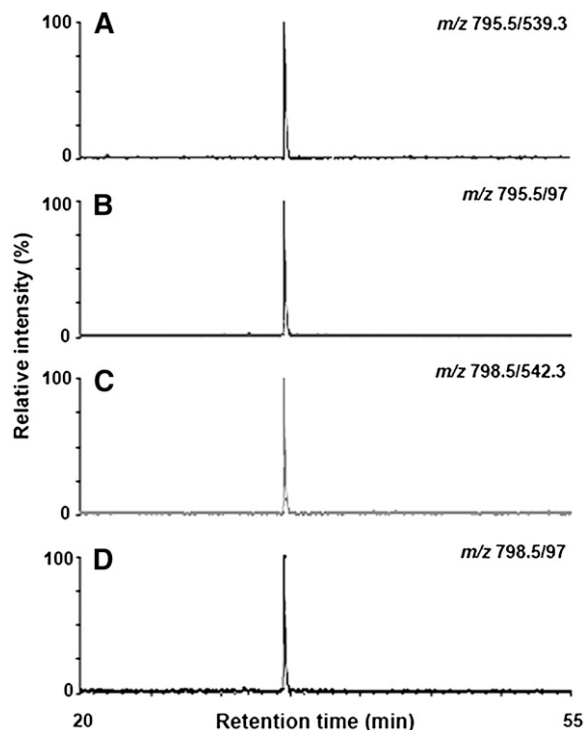
**Fig. 3.** Purity of mouse testis lipid extracts. Top panel: HPTLC of total mouse testis lipids (lanes a and c) and partially purified SGG (lanes b and d). Plates were treated with Coomassie Blue (a and b) and Azure A (c and d). Bottom panel: Sulfate ( $m/z$  97) parent ion electrospray tandem spectra of mouse total testis lipids (A) and partially purified SGG (B).

than 90% of the total ion current (Fig. 3, bottom panel, B); this spectrum was even somewhat cleaner than the corresponding spectrum of the highly purified pig testis SGG (Fig. 2, bottom panel, B). Using the amount of C16:0/C16:0 SGG to calculate the lipid loss during the partial purification process of the mouse testis sample, this value was 16% (Table 1), similar to that seen in the pig testis purification sample (13%). Consequently, the 4.6-fold difference of the amounts of Azure A-reactive lipids over C16:0/C16:0 SGG in the par-

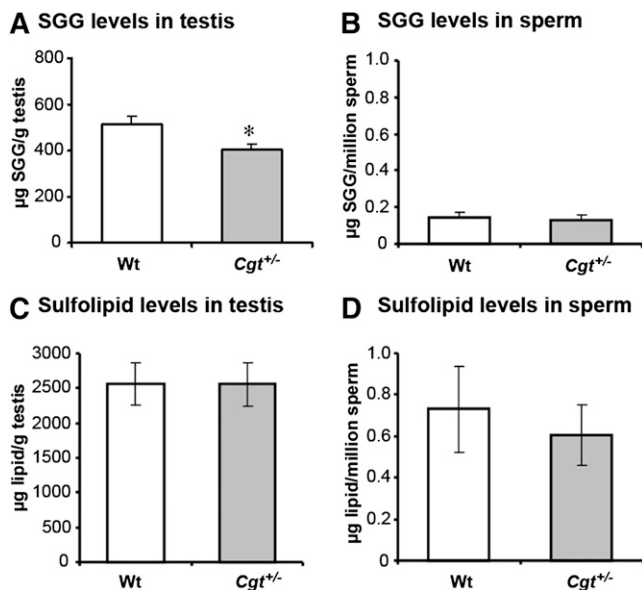
tially purified mouse testis sample was not ascribed to the high levels of other sulfolipids. While the exact reason for this great discrepancy is still unknown, it was possible that there existed a significant amount of anionic lipids that reacted with Azure A in this mouse testis sample. These results highlight the caution that should be exercised when the colorimetric assay is used for precise sulfolipid quantitation. In contrast, the clarity of the MRM signals for C16:0/C16:0 SGG and the internal standard, even when working with total crude lipid extracts (Fig. 4), underscored the use of the MRM method.

#### Levels of C16:0/C16:0 SGG in $Cgt^{+/-}$ mouse testes and sperm

CGT is the initial enzyme in the SGG biosynthetic pathway that transfers galactose onto the lipid backbone of SGG (1-*O*-alkyl-2-*O*-acyl-*sn*-glycerol).  $Cgt$  null males do not contain testicular SGG, and their spermatogenesis is arrested at the pachytene stage (11).  $Cgt^{+/-}$  males, however, sire offspring and are routinely used for colony breeding, although it is not clear whether these heterozygous males are subfertile and whether they possess SGG at levels higher than 50% of the wild-type values. Therefore, it is important to know whether the level of SGG, especially the main molecular species C16:0/C16:0, is similar to that of the wild-type animals. LC-MS/MS MRM analyses re-



**Fig. 4.** MRM chromatograms of C16:0/C16:0 SGG in the total mouse testis lipid extract and the deuterated SGG ( $^2H_3$ -SGG) internal standard. Parent to fragment ion transitions included two for both SGG (A and B) and  $^2H_3$ -SGG (C and D) ( $m/z$  97 for both SGG and  $^2H_3$ -SGG,  $m/z$  539.3 for SGG, and  $m/z$  542.3 for  $^2H_3$ -SGG). The peak heights in arbitrary units for the signals in traces A, B, C, and D were 1526, 3094, 1348 and 1530, respectively. For quantitation, the average of the values obtained for the two transitions was used.



**Fig. 5.** Levels of SGG and total sulfolipids in testes and sperm of *Cgt*<sup>+/-</sup> mice and their wild-type littermates. SGG was quantified by LC-MS/MS-MRM, and total sulfolipids were quantified by the Azure A assay. Three *Cgt*<sup>+/-</sup> mice and three wild-type littermates were used for SGG quantification. For total sulfolipid quantification, two additional *Cgt*<sup>+/-</sup> and wild-type mice were included. \*Significant difference of the values from *Cgt*<sup>+/-</sup> mice compared with the wild-type values ( $P < 0.05$ ).

vealed that *Cgt*<sup>+/-</sup> mouse testes and sperm contained C16:0/C16:0 SGG at the levels of 78% ( $406.06 \pm 23.63 \mu\text{g/g}$  testis) and 87% ( $0.13 \pm 0.02 \mu\text{g/million}$  sperm) of the corresponding wild-type values ( $516.65 \pm 30.62 \mu\text{g/g}$  testis and  $0.15 \pm 0.03 \mu\text{g/million}$  sperm), respectively (Fig. 5). The lower amounts of C16:0/C16:0 SGG in *Cgt*<sup>+/-</sup> mouse testes were significantly different ( $P < 0.05$ ) from those in the wild-type testes, although the decreased levels of C16:0/C16:0 SGG in sperm of *Cgt*<sup>+/-</sup> males were not significantly different from the wild-type values. In contrast, the levels of total sulfolipids in both testes ( $2,556.72 \pm 312.36 \mu\text{g/g}$  testis) and sperm ( $0.60 \pm 0.14 \mu\text{g/million}$  sperm) of *Cgt*<sup>+/-</sup> mice determined by the Azure A assay were not significantly dissimilar from those of the wild-type mice ( $2,562.95 \pm 308.00 \mu\text{g/g}$  testis, and  $0.73 \pm 0.21 \mu\text{g/million}$  sperm) (Fig. 5).

#### Compensatory expression of the CGT polypeptide

Since the SGG and sulfolipid amounts in testes and sperm of *Cgt*<sup>+/-</sup> mice were more than 50% and, in most cases, very similar to the wild-type values, we asked whether the compensation of the CGT expression occurred at the transcriptional or translational level. The *Cgt* transcript in the testis of *Cgt*<sup>+/-</sup> mice was ~40% of the wild-type level, as expected (supplementary Fig. 1). However, the CGT polypeptide in the *Cgt*<sup>+/-</sup> testis was expressed at more than 80% level compared with the wild-type. The CGT polypeptide in the *Cgt*<sup>+/-</sup> testis also contained enzymatic activity, comparable to the enzyme in the wild-type tissue (supplementary Fig. 1). The results indicated the compensatory mechanism of CGT expression at the translational level in *Cgt*<sup>+/-</sup> testes.

#### Fertility status of *Cgt*<sup>+/-</sup> male mice

Although *Cgt*<sup>+/-</sup> male mice sire offspring, their fertility status may still be reduced compared with wild-type males. Results shown in supplementary Fig. II, however, argued against this possibility. Mating studies in five pairs of wild-type and *Cgt*<sup>+/-</sup> male littermates with wild-type females indicated no significant difference in the litter sizes of the offspring ( $7.4 \pm 0.9$  versus  $8.4 \pm 0.9$  pups/litter) (supplementary Fig. IIA). Sperm from *Cgt*<sup>+/-</sup> males also had the same ability as sperm from wild-type animals to fertilize eggs in vitro (supplementary Fig. IIB). The numbers of caudal epididymal/vas deferens sperm retrieved from *Cgt*<sup>+/-</sup> males were also not different from those collected from wild-type animals ( $21.1 \pm 6.9$  million sperm/mouse versus  $18.5 \pm 4.3$  million sperm/mouse,  $n = 7$ ). In addition, the testis weight of the heterozygous and wild-type animals were similar ( $103.7 \pm 6.3$  versus  $110.1 \pm 2.6$  mg/testis,  $n = 7$ ).

#### DISCUSSION

In this study, we described a simple but precise quantification method for C16:0/C16:0 SGG using LC-ESI-MS/MS MRM with <sup>2</sup>H<sub>3</sub>-C16:0/C16:0 SGG as the internal standard. As expected, the internal standard and unlabeled SGG were virtually indistinguishable on the basis of reverse-phase retention time (Fig. 4) and collisionally activated dissociation conditions (24). With this internal standard, it was possible to accurately quantitate SGG in crude total lipid extracts from tissues and cells without further purification of the samples. Further, the internal standard included in the MRM assay can be used to adjust for losses during sample preparation; this is not possible with the colorimetric procedure. Normally the internal standard is added at the beginning of the procedure, but in this study, the internal standard was added after the preparation of the extracts so a comparison between Azure A and MRM data could be made with identical samples. Expansion of the MRM procedure to include other SGG isoforms would be straightforward and achieved by simple selection of the appropriate parent→fragment ion transitions. In contrast, caution should be exercised when attempting to quantitate SGG in crude lipid extracts using the Azure A assay. Results from the mouse partially purified SGG sample indicated that it contained high levels of Azure A-reactive components that were not sulfolipids (Fig. 3, Table 1). Besides sulfolipids, Azure A is known for its binding to anionic compounds (15). Whereas most phospholipids are nonreactive, polyphosphate phospholipids such as cardiolipin, react with the dye (15, 16). Cardiolipin is present in male germ cells at a level comparable to SGG, and it migrates on the HPTLC plate in close proximity to SGG (14). Preparative HPTLC, involving scraping chromatographed lipids around the SGG band, as used for partially purifying the mouse testis lipid samples, was likely inefficient in removing cardiolipin. In contrast, if the lipid sample is further purified, such as the case for the pig testis samples (Fig. 2), which were chromatographed through a BioSil-A column to remove other lipid classes




including phospholipids, the Azure A and MRM data sets were much more convergent (Table 1). Nonetheless, the Azure A procedure for sulfolipid quantification is much less sensitive than the MRM assay (0.5 nmole versus 1 pmole LOD). In general, the Azure A procedure remains a useful technique for obtaining a global picture of the sulfolipid components on an HPTLC plate.

Offsetting the advantages of specificity and sensitivity is the need for appropriate quantitation standards for LC-ESI-MS/MS-MRM. Both SGG and SGC (commercially available) can be used interchangeably as a standard in the Azure A assay, as both are sulfoglycolipids and elicit similar molar responses in the colorimetric procedure. However, SGC is unsuitable as a quantitative standard in the MRM assay for SGG. SGC is an exceptionally stable gas phase ion, requiring stringent conditions for collisionally activated dissociation compared with SGG (18, 22, 38). In addition, besides the  $m/z$  97 ion fragment, the collision-induced fragments of SGC are different from those of SGG (34). Because we were unable to purify a molecularly homogeneous preparation of SGG from natural sources, it was necessary to chemically synthesize C16:0/C16:0 SGG as a quantitative standard. To use it as an internal standard, it was prepared as  $^2\text{H}_3$ -C16:0/C16:0 SGG by incorporating stable deuterium atoms into the molecule as part of the synthesis scheme (24). The MRM-determined concentration of C16:0/C16:0 SGG in mouse testes was about 550  $\mu\text{g/g}$  testis (Table 1 and Fig. 5), somewhat lower than that described by Tadono-Aritomi et al. (23) (i.e.,  $\sim 640$   $\mu\text{g/g}$  testis), who also used LC-MS/MS MRM for quantification. Their slightly higher value would likely be attributed to the fact that their measurements were performed with purified natural SGG as the quantitative standard, which in our experience is contaminated with low levels of other SGG molecular species.

The availability of LC-ESI-MS/MS-MRM to specifically quantify C16:0/C16:0 SGG allowed assessment of the level of this sulfoglycolipid that is required for maintaining male fertility. *Cgt* null males without the ability to synthesize SGG have their spermatogenesis disrupted and are thus infertile. In contrast, *Cgt*<sup>+/-</sup> males sire offspring, although their detailed fertility status is unknown (11). It is possible that these heterozygous males are subfertile, a condition commonly found in men. However, the results from the mating and in vitro fertilization experiments reported herein argued against this possibility. Sperm from *Cgt*<sup>+/-</sup> males could fertilize mature eggs to the same extent as wild-type sperm. The litter size of offspring generated from mating these heterozygous males with wild-type females was also the same as that produced by wild-type males in the parallel mating experiment. Notably, the C16:0/C16:0 SGG levels in sperm of *Cgt*<sup>+/-</sup> males were indistinguishable from those in wild-type sperm. Our accumulated studies indicate the significance of sperm surface SGG in egg interactions (1, 8); it has direct affinity for the ZP and also acts as a major participant in the formation of lipid rafts, which are ZP binding platforms on the sperm head surface (5, 7, 10). Therefore, the normal amounts of C16:0/C16:0 SGG would allow uncompromised function of sperm from *Cgt*<sup>+/-</sup> males.

In contrast, the amounts of C16:0/C16:0 SGG in testis was 78% of the wild-type values; however, spermatogenesis in these animals occurred at the same rate as in the wild-type, as revealed by the same numbers of sperm present in the epididymis and vas deferens and the same testicular weights. These results indicated that about 80% of normal testicular C16:0/C16:0 SGG is sufficient for maintaining spermatogenesis. SGG biosynthesis occurs mainly in the primary spermatocytes (39, 40). During spermiogenesis, when round spermatids become polarized and transformed into elongated spermatids and then testicular sperm, membrane reorganization, including the shedding process of residual bodies, takes place. Mature sperm would likely have reduced levels of SGG, compared with round testicular germ cells, as a result of membrane shedding. It is possible that the shedding of SGG-containing membranes occurred with slightly reduced efficacy in round spermatids of *Cgt*<sup>+/-</sup> males, thus correcting SGG levels in their testicular sperm to the normal values. However, both elongated spermatids and testicular sperm were minor components in the total testis homogenate, and the reduced C16:0/C16:0 SGG levels observed would most likely reflect the decreased lipid levels in round testicular germ cells.

Our results suggest that compensatory translation of active CGT enzyme would be one factor contributing to the 78% SGG expression in the testis of *Cgt*<sup>+/-</sup> mice, although the *Cgt* transcript in the heterozygous testis was only half of the wild-type level. Our unpublished results also reveal that the enzymatic activity and polypeptide level of ASA, the first enzyme in the SGG degradation pathway, in the *Cgt*<sup>+/-</sup> testis were not different from those in the wild-type testis, suggesting that SGG desulfation was not affected in these heterozygous mice. It is still unclear why the expression of C16:0/C16:0 SGG in *Cgt*<sup>+/-</sup> testis was 20% lower than the wild-type levels. CGT's substrates, palmitylpalmitoylglycerol (PPG) and UDP-Gal, might be present at reduced levels in the *Cgt*<sup>+/-</sup> testis. The 50% expression of the *Cgt* transcript might trigger the slow-down of PPG biosynthesis and/or acceleration of its degradation. In the same vein, the expression of the enzyme downstream to CGT in the SGG biosynthetic pathway [i.e., cerebroside sulfotransferase (CST)] may be interlinked with the level of the *Cgt* transcript, and a reduced level of the CST expression and activity could also result in the 78% level of SGG in *Cgt*<sup>+/-</sup> mice. In addition, PPG and/or UDP-Gal might have been shunted to alternative synthesis pathways of other sulfated glycolipids to compensate for the reduced C16:0/C16:0 SGG levels, a situation similar to that observed in the kidney of *Cgt* null mice (41). All these possibilities are ongoing studies in our laboratory. Regardless, the compensation of SGG biosynthesis observed in our study is in accordance with that of other structural lipids such as phosphatidylcholine (PC), which has two synthetic pathways, one utilizing phosphatidylethanolamine methyltransferase (PEMT) and the other CTP-choline cytidyltransferase. Mice genetically null of *Pemt* still possess the same PC levels in their liver as wild-type animals due to the compensatory increase in the CTP-choline cytidyltransferase activity (42). Structural lipids are important for maintaining the integrity of the cell membranes and their microdomains, such as lipid rafts, and

compensatory mechanisms have likely evolved to maintain their net biosynthesis as a self-preserving mechanism. 

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