Intact sphingomyelin biosynthetic pathway is essential for intracellular transport of influenza virus glycoproteins

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Intact sphingomyelin biosynthetic pathway is essential for intracellular transport of influenza virus glycoproteins

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Edited by Kai Simons, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, and approved March 4, 2013 (received for review November 14, 2012)

Cells genetically deficient in sphingomyelin synthase-1 (SGMS1) or blocked in their synthesis pharmacologically through exposure to a serine palmitoyltransferase inhibitor (myriocin) show strongly reduced surface display of influenza virus glycoproteins hemagglutinin (HA) and neuraminidase (NA). The transport of HA to the cell surface was assessed by accessibility of HA on intact cells to exogenously added trypsin and to HA-specific antibodies. Rates of de novo synthesis of viral proteins in wild-type and SGMS1-deficient cells were equivalent, and HA negotiated the intracellular trafficking pathway through the Golgi normally. We engineered a strain of influenza virus to allow site-specific labeling of HA and NA using sortase. Accessibility of both HA and NA to sortase was blocked in SGMS1-deficient cells and in cells exposed to myriocin, with a corresponding inhibition of the release of virus particles from infected cells. Generation of influenza virus particles thus critically relies on a functional sphingomyelin biosynthetic pathway, required to drive influenza viral glycoproteins into lipid domains of a composition compatible with virus budding and release.

lipid raft | flu assembly | sortagging | virus–host interaction

Release of newly assembled influenza virions requires not only a proper mixture of the viral constituents themselves but also relies on host factors such as endoplasmic reticulum (ER) components, glycosyltransferases, and factors that determine intracellular vesicular transport. Some of these host factors might serve as targets of new antiviral strategies (1). Given the tendency of viruses to acquire resistance under selective pressure exerted by the immune system or imposed by antiviral drugs (2–4), host factors are more likely to escape the emergence of resistance. Genome-wide RNAi screens designed to identify such host factors are predicated on this notion, as exemplified for HIV and influenza virus in several recent reports (5–7). These types of genetic approaches readily establish the involvement of particular proteins or other template-encoded products in viral biogenesis. In contrast, the role of complex carbohydrates and lipids is more difficult to discern, precisely because these are not themselves template-encoded, and rather are the end products of complex biosynthetic pathways. Although it is straightforward to alter by mutation a viral nucleic acid and the products it encodes, it is more difficult to selectively modify the carbohydrate or lipid composition of a virus without affecting many host functions in the process.

Enveloped viruses incorporate host components into their membranes for the construction of new infectious particles. The lipid composition of newly formed virions resembles that of the membrane compartments from which they bud. Vesicular stomatitis virus and influenza virus, which bud from the basolateral and apical domains of polarized epithelial cells, respectively, report on the lipid composition of these membrane domains (8, 9). The targeting signals carried by viral membrane glycoproteins, together with the propensity of viral membrane proteins to interact preferentially with certain classes of (glyco)sphingolipids, help determine the characteristics of the membrane domain(s) from which new particles originate (8).

The influenza virus envelope contains two major membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA), along with the M2 protein (10, 11). Assembly of new virus particles requires the coalescence of HA and NA in membrane domains of distinct lipid composition, with M2 proposed to play a later role in membrane scission (12) to achieve particle release. High local concentrations of cholesterol, sphingomyelin (SM), and glyco-sphingolipids impart on these membrane microdomains—also called “rafts”—unique biophysical properties, such as enhanced resistance to extraction by nonionic detergents (13). The debate about the physical size, lifetime, and dynamic behavior of these lipid rafts is unlikely to subside anytime soon (14, 15), nor is it clear whether these are the only membrane specializations controlled by lipid composition.

The role of lipid rafts in cellular processes is a matter of record (16, 17), and perturbation of the homeostasis of its major component, cholesterol, affects membrane behavior (14). Physical extraction of cholesterol with methyl-β-cyclodextrin and/or treatment of cells with drugs that inhibit cholesterol metabolism are common strategies used to perturb cellular pools of cholesterol (18, 19). The use of these compounds is prone to causing off-target effects, and therefore independent approaches, such as genetic manipulation of lipid biosynthetic pathways, are indispensable to extend these observations. Evidence for the role of lipid rafts in viral glycoprotein transport comes mainly from studies that target cholesterol levels. Even though SM is a key component of lipid rafts, its exact role in protein transport and viral biogenesis remains largely unknown. Here we describe the consequences of a defect in SM synthesis caused by inactivation of the SM synthase-1 (SGMS1) gene, as brought about by insertional mutagenesis (20) and pharmacological blockade (21). SGMS1-null cells and their wild-type counterparts are susceptible to infection with influenza and show similar patterns of glycoprotein maturation in transit from the ER through the Golgi apparatus, but SGMS1-deficient cells and myriocin-treated Madin–Darby canine kidney (MDCK) cells show a major delay in maturation and release of new virus particles.


The authors declare no conflict of interest.

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Results

Characterization of SGMS1-Deficient KBM7 Cells. SGMS1 catalyzes the transfer of phosphorylcholine from phosphatidylcholine (PC) onto ceramide to produce SM (Fig. 1A); SGMS1 is the major SM synthase in mammalian cells (22). To examine the role of SM in influenza virus infection, we used SGMS1GT (SGMS1-gene trap), a cell line isolated in a screen in a human haploid cell line selected for resistance to cytolethal distending toxins produced by various pathogenic bacteria (20, 23). SGMS1 transcripts are present in the parental cell line KBM7 but are absent from SGMS1GT cells, as shown by RT-PCR (Fig. 1B). We determined the ability of these cells to synthesize SM by metabolic labeling of KBM7 and SGMS1GT cells with the SM precursor N-methyl-[14C]-choline. We extracted total lipids followed by TLC and autoradiography. SGMS1GT cells showed reduced SM synthesis activity (~15%) compared with the KBM7 cells (Fig. 1 C and D). The residual activity is due to the second enzyme, SGMS2, which accounts for 10–20% of SM synthase activity in mammalian cells (24). We found no differences in the synthesis of [14C]-PC between SGMS1GT and KBM7 cells (Fig. 1 C and D).

SGMS1-Deficient KBM7 Cells Have Significantly Reduced Levels of SM. To assess the contribution of SGMS1 to sphingolipid content at steady state, we determined the lipid composition of SGMS1GT and KBM7 cells using liquid chromatography–tandem mass spectrometry. We find that SGMS1GT cells have ~20% of total cellular SM content compared with KBM7 (Fig. 1E), which corroborates our metabolic labeling experiments (Fig. 1 C and D). Lipid analysis of cells that were cultured for more than 2 wk in delipidated media showed a similar reduction of SM level. Hence, the remaining SM produced in SGMS1GT cells must result from the activity of SM synthase-2 (SGMS2). The decrease in SM levels in SGMS1GT cells was accompanied by a twofold increase of its precursor, ceramide (Fig. 1E). Ceramide is also a precursor for the synthesis of lactosylceramide (LacCer) and glucosylceramide (GlcCer). In SGMS1GT cells the level of LacCer increased fourfold, whereas the level of GlcCer increased twofold compared with KBM7 cells (Fig. 1E). The species of SM present in KBM7 and SGMS1-deficient cells were indistinguishable (Fig. S1). Relative to KBM7 cells, PC levels were higher, whereas phosphatidylethanolamine (PE) levels were lower in SGMS1GT cells (Fig. 1F). Cholesterol content and overall phosphate levels remain unchanged (Fig. 1G). Combined, our data show that SGMS1GT cells have significantly reduced levels of SM compared with the parental KBM7 line, consistent with the known defect in SM synthesis.

SGMS1-Deficient Cells Are Defective in Influenza Virus Production. To examine the role of SM in the context of virus infection, we first assessed the extent to which SGMS1GT cells support an infection with influenza virus. We infected SGMS1GT and KBM7 cells at a multiplicity of infection (MOI) of 0.1. At 24 h after infection, cells were pulse-labeled with [35S]-methionine/cysteine. We analyzed total cell lysates by SDS/PAGE and autoradiography. We detected slightly more newly synthesized host proteins in SGMS1GT compared with KBM7 cells (Fig. 2A). This suggests a possible defect in viral production and in reduction of host protein synthesis in SGMS1GT cells, which therefore may be less able to sustain virus replication. The major viral proteins detected correspond to nucleoprotein (NP) and M1 (Fig. 2A). This led us to examine virus production by SGMS1GT cells. To this end, KBM7 and SGMS1GT cells were infected at an MOI of 0.1, and at 24 h after infection the media was collected and a fluorescent focus assay performed. SGMS1GT cells produced less than half the amount of virus than do KBM7 cells (Fig. 2B).

SGMS1-Deficient Cells Bind Influenza Virus in Amounts Similar to Those Bound by KBM7 Cells. Because SGMS1GT cells show impaired production of influenza virus, we explored the step at which SM is required in the life cycle of the virus. To compare the extent of virus binding to KBM7 and SGMS1GT cells, we used a modified strain of influenza virus (HA-Srt), labeled site-specifically in its HA1 moiety with Alexa 647 (25). SGMS1GT and KBM7 cells were incubated with an equal amount of Alexa 647-labeled virus and/or cholera toxin (CTx) for 30 min on ice to prevent endocytosis and analyzed by cytofluorimetry or confocal microscopy. SGMS1GT and KBM7 cells bound similar amounts of labeled virus (Fig. 2 C and E), indicating that SM is not essential for adsorption of influenza virus. Additionally, SGMS1GT cells and KBM7 bound similar amounts of labeled CTx, particularly at lower concentrations of CTx (Fig. 2 D and E).

Influenza Virus HA Transport from the ER to the Golgi Is Not Affected in SGMS1-Deficient KBM7 Cells. Because initial binding of influenza virus to KBM7 and SGMS1GT cells was similar, we next investigated the trafficking of viral glycoproteins through the secretory pathway. In yeast, sphingolipid synthesis is critical for ER-to-Golgi transport of glycosylphosphatidylinositol (GPI)-anchored proteins such as Gas1p (26), but the role of sphingolipids in glycoprotein trafficking in mammalian cells is less clear. We therefore examined the synthesis and maturation of influenza HA in KBM7 and SGMS1GT cells. The HA precursor (HA0) trimerizes and traffics through the secretory pathway, en route acquiring complex-type N-linked glycans in the Golgi

Fig. 1. SGMS1GT cells have reduced levels of SM. (A) Schematic representation of the SM biosynthesis pathway in mammalian cells. SGMS1-deficient KBM7 cells and myricin, an SPT inhibitor (marked in box) show a block in sphingolipid biosynthesis. (B) RT-PCR analysis showing the absence of the SGMS1 transcript in SGMS1GT cells. (C) Autoradiogram of a TLC separation of lipid extracts of KBM7 and SGMS1GT cells metabolically labeled with the SM precursor [14C]-choline. (D) Quantification of the [14C]-SM and [14C]-PC signals from [14C]-choline labeling experiment in C. (E and F) Total lipids were extracted from KBM7 and SGMS1GT cells and subjected to MS/MS. Levels of sphingolipids (E) and glycerophospholipids (F) are expressed as mole percent relative to total lipid analyzed. (G) Total phosphate and cholesterol levels of KBM7 and SGMS1GT cells were determined and presented as mole percent relative to the control. Error bars represent SD of three (D) or two (E–G) independent experiments. Cer, ceramide; PC, phosphatidylcholine; PS, phosphatidyserine; PI, phosphatidylinositol.

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Intracellular HA0 to yield two fragments, HA1 and HA2 (29). Transport of HA from the ER to the Golgi to the cell surface was considerably delayed in SGMS1GT cells (Fig. 3 C and D). At 30 min of chase, most of HA in KBM7 cells had reached the cell surface, whereas only ~5% arrived in SGMS1GT cells. However, at 60 min of chase, the difference was less pronounced, with ~70% of HA arriving at the cell surface of SGMS1 GT cells (Fig. 3 C and D).

We next determined whether SGMS1GT cells have a more general defect in protein transport. We monitored the level of secreted proteins in KBM7 and SGMS1 GT cells. KBM7 and SGMS1 GT cells were labeled for 30 min with [35S]-methionine/cysteine and chased for different times. Proteins secreted into the media were analyzed by SDS/PAGE and autoradiography. As a negative control, we incubated cells at 4 °C to block secretion. There was no difference in the levels of secreted proteins when comparing SGMS1 GT and KBM7 cells (Fig. S2B).

In parallel, we monitored the transport of class I MHC proteins—type I membrane glycoproteins that traffic through the constitutive secretory pathway to reach the cell surface (30). SGMS1 GT cells show no significant difference in the transport of class I MHC (Fig. 3 E and F), as assessed by rate and extent of acquisition of Endo H resistance.

Myriocin is a potent inhibitor of serine palmitoyltransferase (SPT), an enzyme that catalyzes the first and rate-limiting step of de novo sphingolipid biosynthesis (21). Consequently the levels of ceramide—the substrate for SGMS1 and SGMS2—are expected to be significantly lower in myriocin-treated cells. We treated MDCK cells with 100 nM myriocin for 72 h before virus infection. We determined the extent of inhibition of SM synthesis by metabolic labeling with [14C]-serine or [14C]-choline of myriocin-treated and control MDCK cells and visualized the formation of [14C]-SM by TLC. Cells treated with myriocin (27, 28). Transport of HA from the ER to the Golgi apparatus proceeds normally in SGMS1 GT cells, as assessed by the rate and extent of acquisition of Endoglycosidase H (Endo H) resistance (Fig. 3 A and B). Similarly, we found no measurable delay in trafficking of HA from the ER to the Golgi in MDCK cells treated with myriocin, a specific inhibitor of serine palmitoyltransferase (see below and Fig. 4B).

Influenza Virus HA and NA Transport from the Golgi to Cell Surface Is Affected in Cells Defective of SM Biosynthesis. SGMS1 is localized in the Golgi, where it catalyzes the production of SM on the luminal side of the trans-Golgi network (TGN). On the basis of our analysis of SGMS1 GT cells, we hypothesized that a lack of SM in the Golgi compartment might affect membrane organization and hence impede trafficking of HA. To explore this, we infected SGMS1 GT and KBM7 cells with influenza virus. We monitored arrival of metabolically labeled HA at the cell surface by trypsin treatment, which cleaves surface-disposed but not intracellular HA0 to yield two fragments, HA1 and HA2 (29). Transport of HA from the ER to the Golgi to the cell surface was considerably delayed in SGMS1 GT cells (Fig. 3 C and D). At 30 min of chase, most of HA in KBM7 cells had reached the cell surface, whereas only ~5% arrived in SGMS1 GT cells. However, at 60 min of chase, the difference was less pronounced, with ~70% of HA arriving at the cell surface of SGMS1 GT cells (Fig. 3 C and D).

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showed a near-complete blockade of SM production, whereas the biosynthesis of other phospholipids such as [1-14C]-PC and [1-14C]-PS remained unaffected (Fig. 4A).

We monitored the arrival of HA at the cell surface by infecting MDCK cells with a “sortagging” influenza virus (HA-Srt or NA-Srt), which allows site-specific incorporation of label in surface-displayed HA or NA (25). We infected myriocin-treated and control MDCK cells with HA-Srt and at 5 h after infection labeled them with [35S]-methionine/cysteine. At the indicated time points, and subjected to TX-100 extraction on ice. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartment where SM is necessary for the interaction of HA with lipid rafts. At the 0-h time point, almost all HA was found in the ER compartmen...
Fig. 5. Influenza virus HA and NA retains TX-100 solubility upon blockade of sphingolipid biosynthesis. (A) Myriocin-treated and control MDCK cells were infected with influenza virus, and at 5 h after infection, cells were pulse-labeled with [35S]-methionine/cysteine for 10 min, chased for indicated time points, and subjected to cold 1% TX-100 extraction. HA molecules from the soluble (S) and insoluble (P) fractions were recovered using anti-HA serum, analyzed by SDS/PAGE, and autoradiography. (B) Quantification of three independent experiments performed as described in A or using KBM7 and SGMS1GT cells. (C) Myriocin-treated and control MDCK cells were infected with NA-Srt virus that carries the HA-epitope tag in addition to a sortase recognition motif. At the indicated time after infection, cells were subjected to cold TX-100 extraction. NA molecules from the soluble and insoluble fractions were detected by Western blotting using anti-HA epitope tag-HRP. (E) Quantification of three independent experiments performed as described in D.

Discussion

The assembly of influenza virus particles requires not only viral components themselves but also many host factors essential for glycoprotein maturation, (glyco)lipid synthesis, and vesicular transport. Genome-wide RNAi screens conducted for influenza virus and performed in different cell types have uncovered many host proteins proposed to contribute to virus assembly, but enzymes involved in lipid synthesis have not been identified in such screens (5–7). The SM synthase-1 null cell line (SGMS1GT) used in this study was isolated from the collection of mutants resistant to intoxication by cytolethal distending toxins (20, 23). SGMS1GT cells show a starkly reduced level of SM (15% of wild type) with the concomitant expected increase in its precursors—ceramide and phosphatidylcholine—and in other lipids that use ceramides as a precursor as well. Because of the pronounced preference of influenza virus HA for membrane domains enriched in cholesterol and glycolipids (35), we explored the role of SM content as a parameter that contributes to assembly of new influenza virions. Evidence for the role of lipid rafts in HA and NA trafficking mostly relies on cholesterol depletion by methyl-β-cyclodextrin (18, 31, 36). Although SM is believed to be a major component of rafts, scant evidence exists for a direct role of SM on HA/NA intracellular transport. In addition, contradictory reports on the effect of cholesterol depletion on influenza virus budding indicate that this question is far from solved (37). To this end we used SGMS1GT cells and myriocin, a compound that blocks SM synthesis. Interference with SM synthesis does not affect binding of influenza virus to the cell surface, and only minimally affects the ability of the virus to infect the cell.

extractability of NA, we probed for NA at various time intervals after infection. At 2 h after infection we found NA to be soluble in cold TX-100 both in myriocin treated and untreated MDCK cells, presumably still residing in the early compartments of the secretory pathway. At 6 h after infection, a large fraction of NA in mock-treated MDCK cells becomes insoluble in cold TX-100, whereas in myriocin-treated cells the bulk of NA remains soluble (Fig. 5 D and E).

Methods

Virus Propagation, Infection, and Fluorescent Focus Assay. MDCK cells were cultured in DMEM supplemented with 10% serum, whereas KBM7 cells were grown in Iscove's modified Dulbecco's media (IMDM) with 10% serum. Influenza virus A/WSN/33 was propagated in MDCK cells. Infection of KBM7, SGMS1GT, and MDCK cells was performed as follows: cells were washed twice with PBS and subsequently incubated with virus diluted in PBS/0.2%/1 μg/mL of trypsin at room temperature for 30 min at the given MOI. Cells were then washed once in PBS to remove unbound virus and then placed into fresh DMEM supplemented with 0.2% BSA and 1 μg/mL of trypsin and incubated at 37 °C, 5% CO2. For quantifying virus titer, MDCK cells were infected with serial dilutions of supernatant. After infection, cells were washed and then incubated in 0.8% agar/MEM overlay. After 2 d, the overlay was removed, cells were fixed, and virus foci were detected via immunofluorescence using an NP-FITC antibody.

Myriocin Treatment of MDCK Cells. MDCK cells were either mock treated (only DMSO) or treated with 0.5 μg/mL myriocin (in DMSO; Sigma Aldrich) for 72 h, after which cells were infected with influenza virus. Reduced levels of SM in myriocin-treated cells, was verified by lipid analysis as described. Bio- synthetic labeling of viral proteins was unaffected in myriocin-treated cells as observed by total radiolabel incorporated.

Pulse Chase Experiments, Sortase Labeling, and Glycosidase Digestion. MDCK cells were grown in 10-cm culture dishes and infected with either wild-type influenza virus WSN or engineered HA-Srt or NA-Srt virus (25) at an MOI of 0.5 for 4 h. In addition to sortase recognition sequence (LPETG), NA-Srt
cells were grown in suspension in 24-well plates to a density of 2 x 10^6 cells per well. MDCK cells were infected and recounted in methionine- and cysteine-free DMEM and starved for 45 min at 37 °C followed by a 10-min pulse labeling with [35S]-methionine/cysteine at 0.77 μCi/mL and chase in complete media for 0, 30, and 60 min. Where indicated, 1 μg/mL trypsin was added in the chase media. To probe for surface exposed HA at indicated time points during the chase, cell-surface HA molecules were labeled by SDS/PAGE and autoradiography using DMSO/PPO (2,5-diphenyloxazole) and exposure to Kodak XAR-5 film. Densitometric quantification of radiograms and immunoblots were performed using Photoshop/Image J software.

TX-100 Extraction. TX-100 extraction was performed according to refs 13 and 35. For HA extraction, virus-infected cells were pulse-labeled with [35S]-methionine/cysteine at 0.77 μCi/mL for 10 min and chased for different time points. Cells were harvested, washed once with cold PBS, and resuspended in 0.5 mL of ice-cold Heps-plus buffer (25 mM Heps [pH 7.4], 150 mM NaCl, 1 mM EDTA, and a protease inhibitor mixture (Roche)). Extraction was initiated by addition of 0.5 mL 2% (w/v) TX-100 in Heps-plus buffer (1% TX-100 final concentration) for 30 min on ice. To separate the soluble from the insoluble fraction, cells were centrifuged at 12,000 x g in an Eppendorf microfuge for 20 min at 4 °C. The pellet fraction was solubilized in a Heps-plus buffer containing 1% digitonin with vigorous vortexing, and HA was immunoprecipitated from both fractions using anti-HA serum and analyzed by SDS/PAGE and autoradiography. To determine the detergent solubility of NA protein, cells were infected with NA-Srt virus (25). At different times after infection, cells were harvested and TX-100 extraction was done as described for HA. The pellet fraction of NA was solubilized in SDS sample buffer, and the amount of NA in soluble and insoluble fractions was detected by Western blotting. The insoluble fraction was calculated as the percentage relative to the total fraction (soluble and insoluble).

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Supporting Information

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SI Methods

Antibodies. Antiserum specific for influenza was obtained from mice immunized with influenza virus A/WSN/33. Antiserum against the HA protein of influenza was obtained from trans-nuclear mice with B cells secreting an IgG2b specific for HA. NeutrAvidin agarose beads were from Fisher Scientific.

Analysis of Cell Supernatants. Cells were infected with influenza virus, pulse-labeled with [35S]-methionine/cysteine, and chased as described above. At indicated time points, supernatant was collected, and residual cells were removed by centrifugation. For measuring total levels of radiolabeled virus, the supernatant was incubated with chicken erythrocytes for 1 h at 4 °C with gentle agitation. Virus bound to erythrocytes were collected by centrifugation, washed once with PBS and lysed in SDS sample buffer, and resolved by SDS/PAGE followed by autoradiography.

Lipid Metabolic Labeling and TLC Analysis. Approximately 2 × 10^6 KBM7 and SGMS1^{GT} cells or MDCK cells grown in six-well dishes were labeled with 3 μCi/mL of N-methyl-[3H]-choline (Thermo Scientific) or 3-I-[3H]-serine (Perkin-Elmer) for 4 h in Opti-MEM at 37 °C. Cells were washed twice in PBS and then subjected to lipid extraction following the Bligh and Dyer method (1). The methanol/chloroform extracts (lower face) were dried under a gentle stream of nitrogen and then in chloroform, methanol, 25% ammonia solution (50:25:5, vol/vol/vol). Radiolabeled lipids were detected on a PhosphorImager (Fujiﬁlm BAS-2500) using Image Reader BAS-2500 V1.8 (Fujifilm) and quantified using Quantity One software (Bio-Rad).

Lipid Mass Spectrometry Analysis. Lipid extracts from KBM7 and SGMS1^{GT} cells were prepared according to Foleh et al. (2) but without the salt. Crude lipid extracts were evaporated under N2, dissolved in chloroform/methanol (1:2), and supplemented with internal standards for sphingomyelin (SM), phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylinositol, ceramide, and GlecER as previously described (3). Phospholipid concentration was determined by phosphate analysis and sphingolipid concentration, and cholesterol concentrations were determined according to Tafesse et al. (4).

Flow Cytometry Analysis and Microscopy. Expression, purification, and labeling of cholera toxin (CTXs) were performed as described in ref. 6. The FACS assays were performed with Alexa 647-labeled influenza virus generated as previously described (7) or with CTX conjugated to GGGK-Alexa 647—specifically at the C terminus of its A1 subunit—using sortase A (6). Equal numbers of KBM7 and SGMS1^{GT} cells were incubated at different multiplicities of infection (MOIs) with Alexa 647-labeled virus (the MOI was determined according to hemagglutination assay) or CTX-Alexa 647 and propidium iodide (PI) for 15 min on ice. The cells were washed with cold PBS supplemented with 1% BSA and subjected to cytoﬂuorometry immediately (FACSCalibur; BD Biosciences). Control samples were prepared using PI only. Only cells negative for PI staining were gated for data acquisition. FlowJo was used to analyze the data. Intensity of fluorescence was measured, and the percent maximum presented in the overlaid histograms. For microscopy, cells were incubated with Alexa 647-labeled influenza virus and Alexa 488-labeled CTX subunit B (Invitrogen) on ice as described above. Images were captured using a confocal microscope with a 63× 1.40 N.A. Plan Apo oil objective (Carl Zeiss).

Phosphatidylinositol-Specific Phospholipase C Assay. EL4 cells grown in RPMI supplemented with 10% FCS were treated either with myricoin or vehicle (DMSO) for 3 d. After washing once with PBS, the cells were subjected to 1 U/mL of Bacillus cereus phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma Aldrich) digestion in Opti-MEM for 1 h at room temperature to release glycosylphosphatidylinositol (GPI)-anchored proteins from the cell surface. Cells were washed three times with PBS, fresh media was added, and the cells were placed at 37 °C. At different times thereafter, cells were collected and stained with anti-Thy1.2-PE and anti-class I MHC-FITC on ice for 20 min and analyzed by FACS as described above.

Fig. S1. SM species of KBM7 and SGMS1GT cells. Total lipids were extracted from KBM7 and SGMS1-null cells, and levels of SM species were analyzed by MS/MS as described in Methods. Levels of SM species are given in mole percent of total lipids analyzed. Error bars, SD of two independent experiments.

Fig. S2. Perturbation of sphingolipid biosynthesis modestly affects surface display of Thy1 but not overall protein secretion. (A) Myriocin-treated and control EL4 cells were subjected to PI-PLC treatment for 1 h at room temperature, washed with PBS, and returned to 37 °C in fresh media. At the indicated time points, cells were collected and costained with anti-Thy1-PE (Upper) and class I MHC-FITC (Lower) and analyzed by FACS. (B) Equal numbers of KBM7 and SGMS1GT cells were pulse-labeled with [35S]-methionine/cysteine for 2 h and chased at 37 °C or kept on ice. Supernatants were collected and analyzed by SDS/PAGE and autoradiography.