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

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.
Intact sphingomyelin biosynthetic pathway is essential for intracellular transport of influenza virus glycoproteins

Fikadu G. Tafesse*, Sumana Sanyala*, Joseph Ashoura, Carla P. Guimaraesb, Martin Hermanssonb, Pentti Somerharjub, and Hidde L. Ploegh*1

*Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge, MA 02142; and Department of Biochemistry and Developmental Biology, Institute of Biomedicine, University of Helsinki, 00014, Helsinki, Finland

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)

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 glycosphingolipids 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 Malden–Darby canine kidney (MDCK) cells show a major delay in maturation and release of new virus particles.


*Corresponding author. E-mail: ploegh@wibm.mit.edu.
This article has supporting information online at www.pnas.orglookup supplemental.

PNAS April 16, 2013 | vol. 110 | no. 16 | 6406–6411
www.pnas.org/cgi/doi/10.1073/pnas.1219909110
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 SGMS1<sup>GT</sup> (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 SGMS1<sup>GT</sup> cells, as shown by RT-PCR (Fig. 1B). We determined the ability of these cells to synthesize SM by metabolic labeling of KBM7 and SGMS1<sup>GT</sup> cells with the SM precursor N-methyl-[<sup>14</sup>C]-choline. We extracted total lipids followed by TLC and autoradiography. SGMS1<sup>GT</sup> cells showed reduced SM synthesize activity (~15%) compared with the KBM7 cells (Fig. 1C 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 [<sup>14</sup>C]-PC between SGMS1<sup>GT</sup> and KBM7 cells (Fig. 1C 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 SGMS1<sup>GT</sup> and KBM7 cells using liquid chromatography–tandem mass spectrometry. We find that SGMS1<sup>GT</sup> cells have ~20% of total cellular SM content compared with KBM7 (Fig. 1E), which corroborates our metabolic labeling experiments (Fig. 1C 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 SGMS1<sup>GT</sup> cells must result from the activity of SM synthase-2 (SGMS2). The decrease in SM levels in SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> cells (Fig. 1F). Cholesterol content and overall phosphate levels remain unchanged (Fig. 1G). Combined, our data show that SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> cells support an infection with influenza virus. We infected SGMS1<sup>GT</sup> and KBM7 cells at a multiplicity of infection (MOI) of 0.1. At 24 h after infection, cells were pulse-labeled with [<sup>35</sup>S]-methionine/cysteine. We analyzed total cell lysates by SDS/PAGE and autoradiography. We detected slightly more newly synthesized host proteins in SGMS1<sup>GT</sup> compared with KBM7 cells (Fig. 2A). This suggests a possible defect in viral production and in reduction of host protein synthesis in SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> cells. To this end, KBM7 and SGMS1<sup>GT</sup> 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. SGMS1<sup>GT</sup> cells produce 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 SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> cells, we used a modified strain of influenza virus (HA-Srt), labeled site-specifically in its HA1 moiety with Alexa 647 (25). SGMS1<sup>GT</sup> 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 cytometry. SGMS1<sup>GT</sup> and KBM7 cells bound similar amounts of labeled virus (Fig. 2C and E), indicating that SM is not essential for adsorption of influenza virus. Additionally, SGMS1<sup>GT</sup> cells and KBM7 bound similar amounts of labeled CTx, particularly at lower concentrations of CTx (Fig. 2D 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 SGMS1<sup>GT</sup> 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 SGMS1<sup>GT</sup> cells. The HA precursor (HA0) trimerizes and traffics through the secretory pathway, en route acquiring complex-type N-linked glycans in the Golgi.
Transport of HA from the ER to the Golgi apparatus proceeds normally in SGMS1GT 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 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 SGMS1GT cells, we hypothesized that a lack of SM in the Golgi compartiment might affect membrane organization and hence impede trafficking of HA. To explore this, we infected SGMS1GT 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 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 SGMS1GT 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 SGMS1GT cells. KBM7 and SGMS1GT 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 SGMS1GT 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). SGMS1GT 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...
We also examined trafficking of NA in myriocin-treated and untreated MDCK cells infected with NA-Srt virus (25), which encodes an NA that carries an HA epitope tag distal to the sortase recognition sequence. Surface-accessible NA was labeled with GGGK-biotin in the presence of sortase A for 1 h on ice. We used this approach because the abundance of NA is far less than that of HA, complicating its detection by metabolic labeling (32). Biotinylated (= surface-disposed) NA was detected in total cell lysate in immunoblots using streptavidin-HRP (Fig. 4E, Upper). Total NA levels (= surface + intracellular) were measured using an HRP-conjugated anti-NA epitope antibody, which does not recognize the HA of the A/WSN/33 virus (Fig. 4E, Middle). We observed a significant delay in the arrival of NA at the cell surface in myriocin-treated cells (Fig. 4 E and F). Sphingolipid biosynthesis is thus required for surface display of both HA and NA.

To determine the effect of myriocin treatment on the level of influenza virus production we infected myriocin-treated and control MDCK cells with the virus and performed pulse-chase experiment at 5 h after infection. At different time points during the chase the supernatants were collected and incubated with chicken erythrocytes to recover radiolabeled virus released into the media. Consistent with the SGMS1GT results, we found a strongly reduced level of viral proteins in the supernatants of myricin-treated MDCK cells (Fig. 4D), indicating that intact sphingolipid biosynthesis is required for efficient production of influenza virus. Collectively, our data show that proper sphingolipid biosynthesis makes a key contribution to surface display of flu glycoproteins and hence virus particle formation and release.

We then determined whether SM depletion disrupts trafficking of endogenous lipid raft-associated proteins by monitoring the rate of the recovery of Thy1, a glycosylphosphatidylinositol-anchored membrane glycoprotein, on the cell surface after its removal by phosphatidylinositol-specific phospholipase C (PI-PLC) digestion. Myriocin-treated and control EL4 cells were treated with PI-PLC for 1 h at room temperature. After extensive washing to remove traces of PI-PLC, cells were returned to 37 °C. At different time points, the cells were costained with anti-Thy1-PE and anti-class I MHC-FITC and analyzed by FACS (Fig S2A). We found only slightly less Thy1 on the cell surface of myriocin-treated cells at 3 h incubation point compared with untreated EL4 cells (Fig S2A, Upper) and no difference with respect to class I MHC levels (Fig S2A, Lower).

**Influenza Virus HA and NA Show Increased Triton X-100 Solubility upon Blockade of SM Biosynthesis.** Influenza virus HA and NA are known to associate with lipid rafts (17, 19, 33), but the role of SM in this process remains largely unknown. Lipid rafts and their associated proteins show impaired solubility in nonionic detergents such as Triton X-100 (TX-100) or Nonidet P-40 at 4 °C (34). Because transport of HA from the TGN to the cell surface is impaired in SGMS1-deficient cells, we examined the solubility of HA in cold nonionic detergent. For this purpose, virus-infected KBM7 and SGMS1GT cells were pulse-labeled with [35S]-methionine/cysteine for 10 min, chased for different time points, and subjected to TX-100 extraction on ice. At the 0-h time point HA resides in the ER, whereas at 2 h the majority of HA localizes to the Golgi/post-Golgi compartment, as assessed by acquisition of Endo H resistance. This helped identify the 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 soluble fraction, both in KBM7 and SGMS1GT cells. However, at the 2-h time point HA remained soluble in cold TX-100 in SGMS1GT cells, whereas in KBM7 cells a far greater fraction was detergent-insoluble (Fig. 5C). Treatment of MDCK cells with myricin also yielded an increased fraction of TX-100 soluble HA (Fig. 5 A and B). Similarly, to measure cold detergent

**Fig. 4.** Myriocin-treated MDCK cells are defective in cell-surface display but not ER to Golgi transport of influenza HA and NA. (A) MDCK cells treated with myricin for 3 d were labeled with [14C]-choline or [14C]-serine for 4 h and lipids were extracted and analyzed by TLC. (B) MDCK cells treated with myricin as described in A were infected with influenza virus at an MOI of 0.5. At 5 h after infection, cells were pulse-labeled with [35S]-methionine/cysteine for 10 min and chased for the indicated time points in the presence of trypsin added to the chase medium. Cells were lysed and HA molecules were recovered using anti-HA serum, subjected to Endo H treatment, and analyzed by SDS/PAGE and autoradiography. (C) MDCK cells treated with myricin as described in A were infected at an MOI of 0.5 with HA-Srt virus, and at 5 h after infection cells were pulse-chased as described in B. At the indicated time points, surface-accessible HA-Srt was labeled with GGGK-biotin in the presence of sortase A for 1 h on ice. Cells were lysed, and biotinylated HA was recovered by affinity adsorption on NeutrAvidin agarose beads. (D) MDCK cells treated with myricin and infected as described in C were pulse-labeled with [35S]-methionine/cysteine for 10 min. At the indicated time points the supernatants were collected and incubated with chicken erythrocytes to recover radiolabeled virus released into the media. Samples were analyzed as described in B. (E) Myricin-treated MDCK cells were infected with NA-Srt virus and at the indicated time points after infection, cells were labeled with GGGK-biotin in the presence of sortase A for 1 h on ice. Cells were lysed, and biotinylated NA (= surface-exposed) was detected by immunoblotting using streptavidin-HRP (E, Upper), and total NA (= surface + intracellular) was detected using anti-NA epitope tag-HRP (E, Middle). (F) Quantification of experiments performed as described in E. Error bars, SD of three independent experiments. *The identity of this protein band is unknown.
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).

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-I null cell line (SGMS1GT) used in this study was isolated from the collection of mutants resistant to intoxication by cytolethal distending toxins (20, 23). Quantiﬁcation of three independent experiments performed as described in A or using KBM7 and SGMS1GT cells (C). (D) 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.

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% FCS at a m.o.i. of 30 to 50 PFU/cell. After infection, cells were washed three times with 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 for 30 min. The overlay was then removed. For quantifying virus titer, MDCK cells were infected with serial dilutions of supernatant. After infection, cells were washed twice and then incubated in 0.8% agar/MEM overlay. After 2 d, virus foci were detected via immunofluorescence using an NF-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 myricin-treated cells, was veriﬁed by lipid analysis as described. Biosynthetic labeling of viral proteins was unaffected in myricin-treated cells as observed by total radiolabel incorporation.

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 m.o.i. of 0.5 for 4 h. In addition to sortase recognition sequence (LPETG), NA-Srt trimerizes in the ER and assumes its native fold as it prepares to exit for the Golgi, a protracted process that involves imposition of the complex disulﬁde-bonding pattern to stabilize the native trimer (38). How a lipid annulus of deﬁned composition is organized around the membrane anchors of HA and, by extension, NA is not known, but interactions might be through palmitoylation (39) and/or through the polypeptide segments interposed between the transmembrane anchors and the luminal portions with well-deﬁned tertiary structure (18, 36). We show that compromised SM synthesis does not interfere with maturation of the N-linked glycans on HA, indicating that the transport of HA from the ER to the Golgi is not dependent on SM. The block in maturation of viral particles in SGMS1GT cells and myricin-treated cells therefore must occur at or beyond the trans-Golgi network.

Consistent with this notion, we found that surface display of viral glycoproteins is affected when sphingolipid biosynthesis is compromised. We established this using three independent criteria: access of cell surface-exposed HA or NA to trypsin and sortase, as well as inhibition of release of virus particles. SM thus contributes to protein sorting from the TGN to the cell surface. The formation of protein carrier vesicles that bud from the TGN to deliver their cargo to the cell surface requires SM (40), and perturbation of SM biosynthesis might therefore affect cargo sorting. SM has affinity for cholesterol and forms lipid microdomains with distinct protein composition (41). Depletion of cholesterol through application of compounds such as methyl-β-cyclodextrin and lovastatin/mevalonate affect trafﬁcking of raft-associated proteins, including GPI-anchored and some transmembrane proteins, such as inﬂuenza HA (42). As shown here, removal of SM is sufﬁcient to cause a viral glycoprotein trafﬁcking defect in its own right. Although SGMS1GT cells showed increased levels of ceramide and the glycosphingolipids LacCer and GlcCer, known to form lipid microdomains (43), they do not compensate for the reduction of SM as a requirement for proper trafﬁcking of HA and NA, suggesting the possible existence of distinct classes of lipid microdomains with distinct biological roles. Overall, our data demonstrate how inﬂuenza virus and possibly other enveloped viruses exploit host factors such as cellular lipids during their life cycle. Interference with these pathways may provide new means of blunting the pathological effects caused by enveloped viruses.
carries an HA epitope tag (25). KBM7 cells (wild type and SGMS1-deficient) were grown in suspension in 24-well plates to a density of 2 × 10^6 cells per well. Cells were infected and resuspended 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 mCi/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 with 250 μM GGGRG biotin and 100 μM SrtA for 1 h at 4 °C. To measure total intracellular HA, MDCK cells were collected, lysed in Tris buffer [150 mM NaCl, 5 mM MgCl2, 25 mM Tris (pH 7.4)] containing 0.5% Nonidet P-40 followed by immunoprecipitation with influenza virus HA-anti serum. For MDCK cells infected with NA-Srt virus, at indicated times after infection, cells were incubated for 1 h with 100 μM SrtA and 250 μM GGGRG biotin. Cells were lysed in buffer containing SDS, and biotinylated HA (anti-HA epitope tag-HRP (3F10). This antibody (3E10) does not recognize the MDCK cells infected with NA-Srt virus, at indicated times after infection, cells were harvested and TX-100 extraction was done as described for class I MHC molecules were recovered using the monoclonal antibody (W6/32) to MHC molecules. Immunoprecipitates were eluted with SDS sample buffer and resolved by SDS/PAGE. Samples were visualized with autoradiography using DMSO/PPO (2,5-diphenyloxazole) and exposure to Kodak XAR-5 film. Densitometric quantification of radigrams and immunoblots were performed using PhotoshopImage 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 mCi/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 μL 2% (w/vol) TX-100 in Heps-plus buffer (1% TX-100 final concentration) for 30 min on ice. To separate the insoluble from the soluble fraction, cells were centrifuged at 12,000 × 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 HA 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 SD 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).

**ACKNOWLEDGMENTS.** This work was supported by National Institutes of Health R01 Awards AI033456 and AI087879. Funding has also been received from The Netherlands Organization for Scientific Research (F.G.T.) and from Sanofi Pasteur.

Supporting Information

Tafesse et al. 10.1073/pnas.1219909110

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 transgenic mice with B cells secreting an IgG2b specific for HA. NeutAvidin 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-[14C]-choline (Thermo Scientific) or 3-I-[14C]-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 flow of N2. Lipids were dissolved in chloroform, methanol (1:2, vol/vol), and separated by TLC, which was developed first in acetone and then in chloroform, methanol, 25% ammonia solution (50:25:6, 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, phosphati-
diylinositol, ceramide, and GlcCer 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). The lipid molecular species and classes were quantified by liquid chromatography-MS as detailed by Hermansson et al. (5) using an Interchrom Lichrosphere diol-modified silica column coupled on line to a Quattro Micro triple quadrupole mass spectrometer (Micromass).

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 phosphatidinositol-specific phospholipase C (PI-PLC) (Sigma Aldrich) digestion in Opti-MEM for 1 h at room temperature to release glycosphingosiphatidinositol (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 costained 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 SGMS1 GT 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 SGMS1 GT 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.