The host-cell restriction factor SERINC5 restricts HIV-1 infectivity without altering the lipid composition and organization of viral particles

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The host-cell restriction factor SERINC5 potently suppresses the infectivity of HIV, type 1 (HIV-1) particles, and is counteracted by the viral pathogenesis factor Nef. However, the molecular mechanism by which SERINC5 restricts HIV-1 particle infectivity is still unclear. Because SERINC proteins have been suggested to facilitate the incorporation of serine during the biosynthesis of membrane lipids and because lipid composition of HIV particles is a major determinant of the infectious potential of the particles, we tested whether SERINC5-mediated restriction of HIV particle infectivity involves alterations of membrane lipid composition. We produced and purified HIV-1 particles from SERINC5293T cells with very low endogenous SERINC5 levels under conditions in which ectopically expressed SERINC5 restricts HIV-1 infectivity and is antagonized by Nef and analyzed both virions and producer cells with quantitative lipid MS. SERINC5 restriction and Nef antagonism were not associated with significant alterations in steady-state lipid composition of producer cells and HIV particles. Sphingosine metabolism kinetics were also unaltered by SERINC5 expression. Moreover, the levels of phosphatidylserine on the surface of HIV-1 particles, which may trigger uptake into non-productive internalization pathways in target cells, did not change upon expression of SERINC5 or Nef. Finally, saturating the phosphatidylserine-binding sites on HIV target cells did not affect SERINC5 restriction or Nef antagonism. These results demonstrate that the restriction of HIV-1 particle infectivity by SERINC5 does not depend on alterations in lipid composition and organization of HIV-1 particles and suggest that channeling serine into lipid biosynthesis may not be a cardinal cellular function of SERINC5.

For efficient replication in natural target cells, HIV has to surmount numerous physical barriers posed by cell-intrinsic restriction factors such as Trim5α, APOBEC3G, CD317/tetherin, Mx2, or SAMHD1 (1, 2). Because their expression is often triggered or enhanced by interferons, restriction factors are considered an essential arm of cell-intrinsic innate immunity. HIV can overcome these barriers by genetic adaptation, preventing the recognition of subviral structures by restriction factors (*e.g.* modification of HIV-1[4] capsid to prevent interaction with human Trim5α) or by expression of viral antagonists that counteract the antiviral activity of restriction factors. With Vif, Vpr, Vpu, and Nef, HIV-1 encodes four accessory proteins that are not essential for virus replication in cell culture but whose role in antagonizing host-cell restrictions to optimize virus spread *in vivo* is increasingly emerging. Although prominent restrictions antagonized by *e.g.* Vif (apolipoprotein B mRNA editing enzyme), Vpr (SLX4), and Vpu (CD317/tetherin) are well established (3–6), it was only recently that serine incorporator 3 (SERINC3) and SERINC5 were identified as host cell restriction factors that potently impair the infectivity of HIV-1 virions and that are antagonized by HIV-1 Nef (7, 8).

Nef is a myristoylated, 25- to 34-kDa protein that, in addition to HIV-1, is encoded by HIV-2 and simian immunodeficiency virus. In the infected host, Nef potently increases virus replication and thus serves as a pathogenicity factor that accelerates disease progression (9–11). Nef affects many central processes in HIV target cells that may contribute to its role in AIDS pathogenesis, including down-regulation of an array of receptors from the surface of infected cells (12, 13), alteration of signal transduction pathways such as T cell receptor signaling,

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This article contains supplemental Table S1.

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[4] The abbreviations used are: HIV-1, HIV, type 1; SM, sphingomyelin; HexCer, hexosylceramide; SP, sphingolipid(s); SERINC, serine incorporator; PS, phosphatidylserine; GP, glycerophospholipid(s); PC, phosphatidylcholine; GL, glycerolipid(s); HCV, hepatitis C virus; PE, phosphatidylethanolamine; APC, allophycocyanin; TfR, transferrin receptor; PG, phosphatidylglycerol; PI, phosphatidylinositol; ST, sterol(s); pacSph, photoactivatable and clickable analog of sphingosine; Nef, negative factor; HPTLC, high performance thin layer chromatography; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DOPE, 1, 2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

(14–20), as well as interference with host-cell actin dynamics and motility (21–28). Nef also increases the infectivity of HIV-1 particles when expressed from proviral DNA in virus-producing cells (29–31). Although this effect is mild in most cell types, production of HIV-1 variants lacking Nef expression (HIV-1ΔNef) results in particles that are up to 100-fold less infectious than WT HIV-1 (32). These strong differences in virion infectivity correlate with high levels of SERINC5 expression, and Nef antagonizes the antiviral activity of SERINC5 under these conditions to restore full HIV-1 infectivity (7, 8). The mechanisms by which Nef antagonizes SERINC5 restriction to HIV infectivity are not fully understood (33). Nef expression reduces cell surface exposure and virion incorporation of SERINC5; however, these effects are not sufficient to fully account for the antagonism, suggesting that Nef can also inactivate virion-incorporated SERINC5 molecules (7, 8, 34).

Although some insight has emerged regarding the mechanism of SERINC5 antagonism by Nef, molecular details about how SERINC5 interferes with HIV infectivity remain elusive. SERINC5 is a member of the SERINC protein family that is conserved from yeast to mammals and encompasses five members that are predicted to contain 10–12 transmembrane domains (35, 36). SERINC5 is efficiently incorporated into HIV particles, and its presence in the virion may be critical for its antiviral activity (7, 8). In support of this model, SERINC5 reduces the efficacy of virus entry at the fusion step, and the Env glycoprotein contains a major determinant of HIV particles for sensitivity to SERINC5 restriction (7, 8, 37). A recent study suggested that the reduced fusogenicity of SERINC5-containing virions reflects a direct inactivation mechanism of the Env glycoprotein by the restriction factor (38), but the underlying molecular mechanism is still unclear. The overall biochemical properties and physiological roles of SERINC proteins are also not well characterized. The only report available to date describes that SERINC proteins, when ectopically expressed in *Escherichia coli*, yeast, and COS-7 cells, associate with key enzymes required for lipid and sphingolipid biosynthesis and incorporate serine into membranes, which coined the name serine incorporator (35). Reflecting that HIV buds from specialized plasma membrane microdomains, its lipid envelope displays a highly specialized lipid composition that is enriched in cholesterol and sphingolipids and that significantly differs from that of bulk plasma membrane (39, 40). This specialized lipid composition is critical for virion infectivity because, *e.g.*, alterations thereof caused by inhibition of sphingolipid biosynthesis significantly reduce the infectivity of HIV-1 particles (39). Considering that serine is an essential building block of sphingolipid biosynthesis and that this process is critical for full infectivity of HIV-1 particles (39), we hypothesized that the antiviral activity of SERINC5 may be linked to modulation of host-cell lipid biosynthesis and/or lipid composition of virus particles. We noted previously that HIV-1 Nef has the ability to slightly modify the lipid and sphingolipid content of HIV-1 particles when produced from the human CD4 T cell line MT-4, which expresses little or no SERINC5 (7, 41), raising the possibility that this activity is emphasized in the presence of SERINC5 and allows Nef to antagonize the restriction. To test these hypotheses, we set out to define the impact of SERINC5 on HIV-1 particle lipid composition, lipid composition and sphingosine metabolism of HIV-producing cells, and lipid organization of HIV-1 virions.

Results

Purification of HIV particles produced in the presence of SERINC5

Ectopic expression of SERINC5 in 293T cells, which express low levels of endogenous SERINC5 that are not sufficient to exert significant antiviral activity (7), potently suppresses the infectivity of *nef*-negative HIV-1 (ΔNef), whereas Nef expression by WT HIV-1 antagonizes particle infectivity restriction by SERINC5 (7, 8, 34, 42). We therefore used ectopic expression of SERINC5 in 293T cells to test whether the presence of SERINC5 during the production of HIV particles affects the lipid composition of viral progeny. 293T cells were transfected with proviral plasmids together with an empty control or a SERINC5 expression plasmid (Fig. 1) in a large-scale format. Quantification of reverse transcriptase activity in the cell culture supernatants revealed that comparable amounts of virus particles were produced under all conditions (Fig. 1*A*). SERINC5 markedly reduced the relative infectivity of HIV-1 ΔNef particles, whereas expression of Nef from HIV-1 WT counteracted the antiviral activity of SERINC5 (Fig. 1*B*). Virus particles were purified from these cell culture supernatants via velocity gradient centrifugation using a protocol that yields HIV particles without substantial contamination with extracellular vesicles (39, 41). These concentrated virus preparations contained significant amounts of SERINC5 in the case of HIV-1 ΔNef, whereas only very low levels of the restriction factor were detectable in preparations of HIV-1 WT (Fig. 1*C*). SDS-PAGE and silver staining of these virus preparations confirmed that HIV-1 p24 was the main protein component of these preparations, which displayed comparable purity (Fig. 1*D*).

SERINC5 does not alter the lipid composition of HIV-producing cells or HIV particles

We next analyzed these HIV particle preparations and the corresponding producer cells by quantitative lipid mass spectrometry. To this end, we subjected purified viral particles and whole producer cells to lipid extractions in the presence of internal lipid standards. Lipid profiles of HIV-1 particles (Fig. 2*A*, primary data in supplemental Table S1) and producer cells (Fig. 2*B*) were determined by a bottom-up lipidomics approach based on precursor ion and neutral loss scanning for lipid class–specific fragments (43). In line with previous reports (39, 40, 44–47), HIV-1 WT particles were enriched in cholesterol, sphingomyelin (SM), hexosylceramide (HexCer), and phosphatidylserine (PS) compared with the producing cells (Fig. 2, *A* and *B*). No significant differences in lipid composition were observed between HIV-1WT and ΔNef virions (Fig. 2*A*, major and rare lipid classes are shown in the *left* and *right panel*, respectively; compare *light blue* and *dark blue columns*). Presumably reflecting differences in the overall lipid composition of the cell lines used, these particles, produced from 293T cells, did not display the Nef-mediated enrichment in SM observed previously in virions produced from MT-4 cells (40, 41). Considering the suggested potential of SERINC proteins to feed

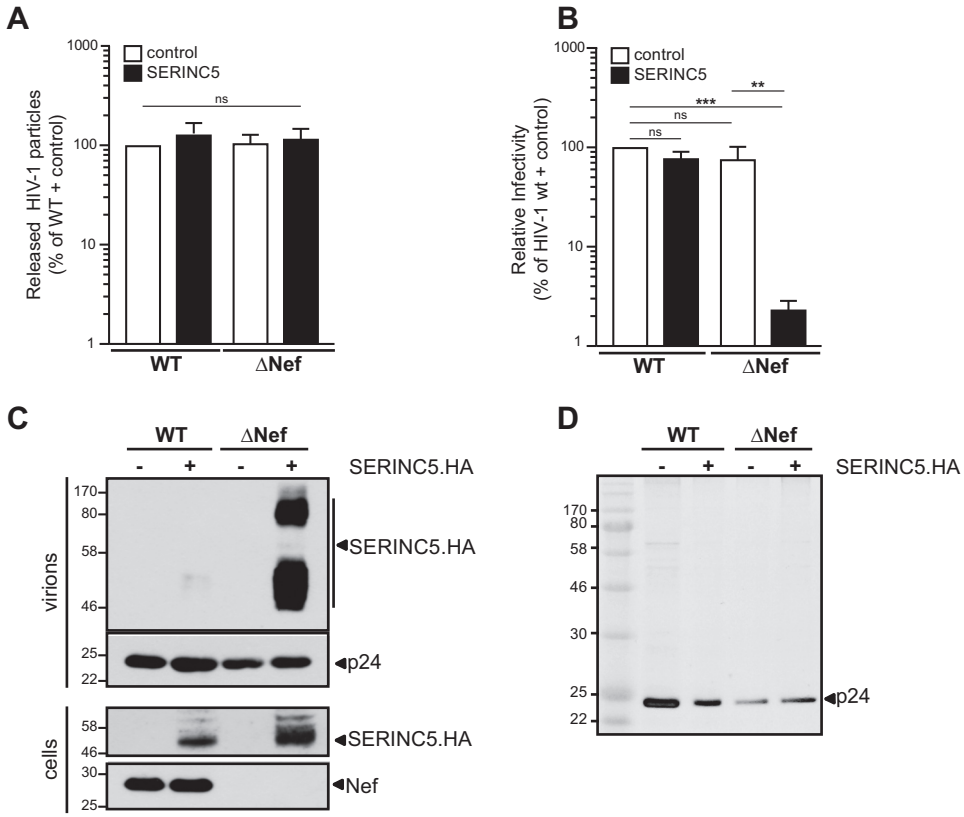


Figure 1. SERINC5 restriction to HIV-1 infectivity and antagonism by Nef. *A*, quantification of the RT activity of OptiPrep-purified virus particles. HIV-1 particles were produced in 293T cells in the absence or presence of SERINC5.intHA, respectively, and purified via velocity gradient centrifugation on an OptiPrep gradient. RT activity (microunits RT) was determined via SYBR Green I— based, product-enhanced reverse transcriptase analysis. Depicted are means of three independent experiment ± S.D., represented relative to HIV-1 WT + control (percent), which was arbitrarily set to 100%. *ns*, no statistical significance. *B*, relative infectivity of the HIV-1 particles analyzed in *A*. To determine relative virion infectivity, TZM-bl reporter cells were infected, and infection efficiency was assessed by measuring relative light units and normalized to virus release. Depicted are means of three independent experiment ± S.D., represented relative to HIV-1 WT + control (percent), which was arbitrarily set to 100% ($n = 3$). **, $p < 0.01$; ***, $p < 0.001$. *C*, Western blot analysis of HIV-1–producing cells and OptiPrep-purified virus particles. Shown is immunodetection of SERINC5.intHA, Nef, and HIV-1 capsid (p24). *D*, silver staining of an SDS-PAGE of OptiPrep-purified virus particles. HIV-1 capsid (p24) is readily detected in all samples.

serine into sphingolipid (SP) biosynthesis, we expected that expression of SERINC5 during virus production may affect levels of host-cell and/or virion lipids that require serine during biosynthesis, such as SM, HexCer, and PS. However, and irrespective of the presence of Nef, expression of SERINC5 during virus production had no significant impact on the abundance of individual lipid classes in virions (Fig. 2*A*) or producer cells (Fig. 2*B*). Likewise, the overall category profile, including glycerophospholipids (GP), SP, and sterols (ST), was indistinguishable in virus producer cells and virions for all tested conditions (Fig. 2*C*).

Although overall lipid class profiles remained unchanged by the presence or absence of SERINC5 during virus production, differences might occur for individual lipid species within lipid classes. To test for changes at the level of molecular lipid species compositions, we first compared their distributions on a global level. In agreement with previous reports (39, 40, 44–47), we observed, for HIV-1 particles, an increase relative to producer cells in GP, predominantly with a total of 30 and 32 fatty acyl carbon atoms (Fig. 3*A*, compare *gray* and *blue columns*, *e.g.* for phosphatidylcholine (PC), *e.g.* 30:0; 32:0, O-32:0). This increase was at the expense of species with a length of 34 and 36 carbons (*e.g.* PC 34:1, 36:2; O-34:1; O-36:2). Again, these profiles remained unchanged by the presence of Nef and/or SERINC5. In turn, the global length distribution of GP and SP species was overall similar in producer cells and virions under all conditions tested. As the only exception, GP-34 was slightly more abundant in cells than in virions (Fig. 3*B*).

Another profound difference between HIV-1 particles and membrane lipids of producer cells is the enrichment of saturated lipid species, contributing to the higher lateral order of particles (39, 40, 48). Accordingly, HIV-1 particles (WT as well as ΔNef) were enriched in GP with saturated fatty acyl moieties (Fig. 3*C*, *left panel*, *e.g.* GP-0), whereas only slight differences between producer cells and HIV particles were observed for the saturation of SP (Fig. 3*C*, *right panel*). For both lipid categories, absence of Nef and presence of SERINC5 did not impact the global GP and SP compositions. Thus, SERINC5 did not influence the partitioning of saturated lipid species or individual lipid classes into the viral membrane envelope.

A striking feature of HIV-1 and some other viruses is the enrichment of dihydrosphingomyelin, such as *N*-palmitoyl-dihdrosphingomeylin (annotated as SM 34:0;2 in Fig. 3*A*, *right panel*) in viral particles compared with producer cell lines (39, 40, 48, 49). We showed previously that dihydrosphingomyelin is not enriched in HIV-1 particles produced from HEK293T cells (47). Consistently, we did not observe an increase in dihy-

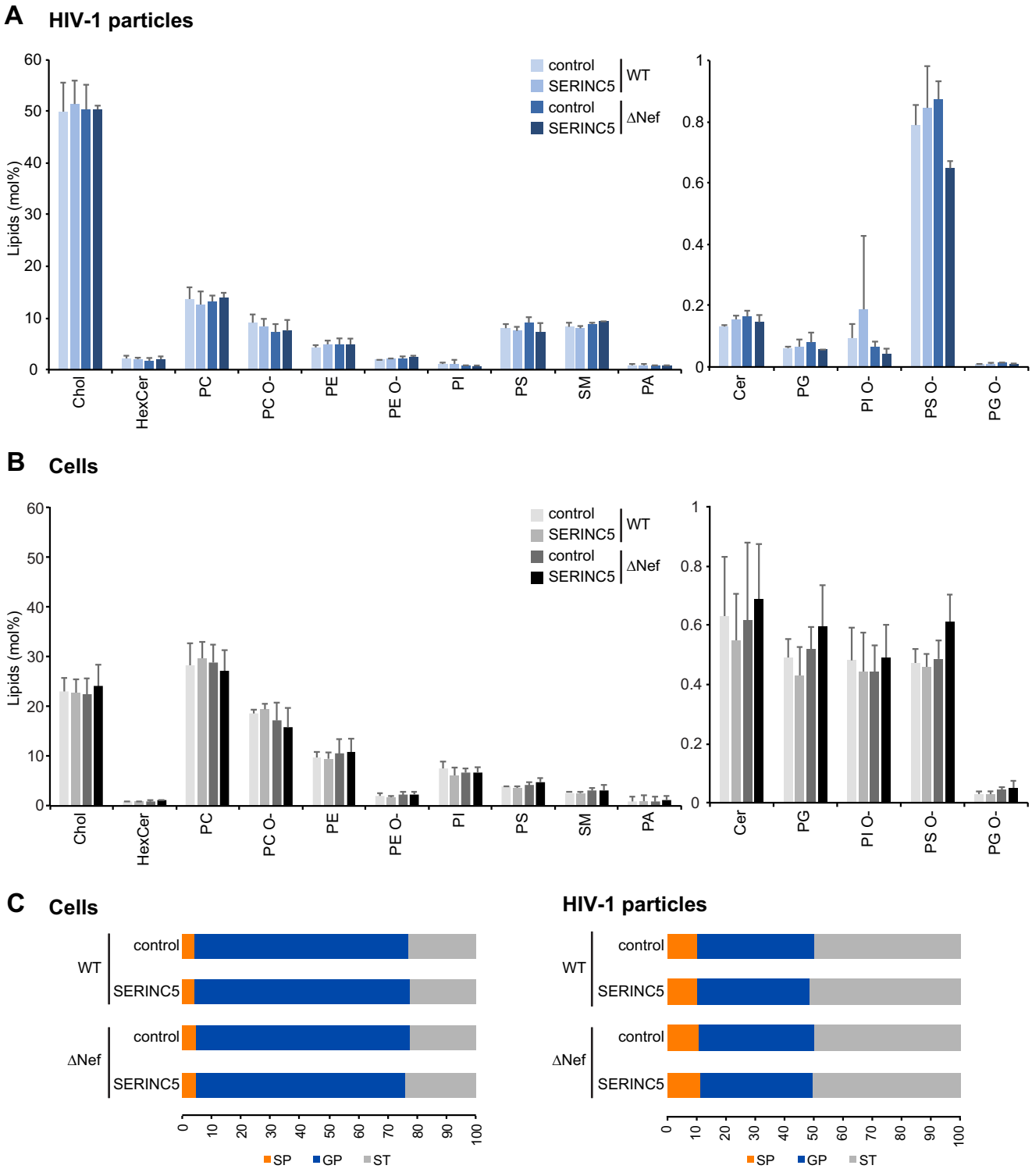


Figure 2. Quantitative lipid analysis of HIV-1–producing cells and purified HIV-1 particles. *A* and *B*, lipid composition of OptiPrep-purified HIV-1_{NL43} WT or ΔNef particles produced in the absence or presence of SERINC5 (*A*) and of the corresponding 293T HIV-1–producing cells (*B*). Lipid classes are standardized to all lipids measured. *Cer*, ceramide; *Chol*, cholesterol; *-O*, ether or odd-numbered fatty acyl residue. Data are presented as mean ± S.D. of three independent experiments, except for ΔNef particles produced in the presence of SERINC5 ($n = 2$). Note that low-abundant lipid classes are presented in separate graphs on the *right*. *C*, relative contribution of SP, GP, and sterols (ST, cholesterol) to the lipid composition of HIV-1–producing cells and OptiPrep-purified HIV-1_{NL43} WT or ΔNef particles produced in the absence or presence of SERINC5.

drosphingomyelin, neither in HIV-1 WT nor in ΔNef particles produced in the absence or presence of SERINC5 (Fig. 3*A*, *right panel*, compare *light blue* and *dark blue columns* of the 34:0;2 species).

Taken together, expression of SERINC5 did not significantly alter the lipid composition of HIV-producing cells or the resulting virus particles. We conclude that, in the experimental system used, restriction of HIV particle infectivity by SERINC5

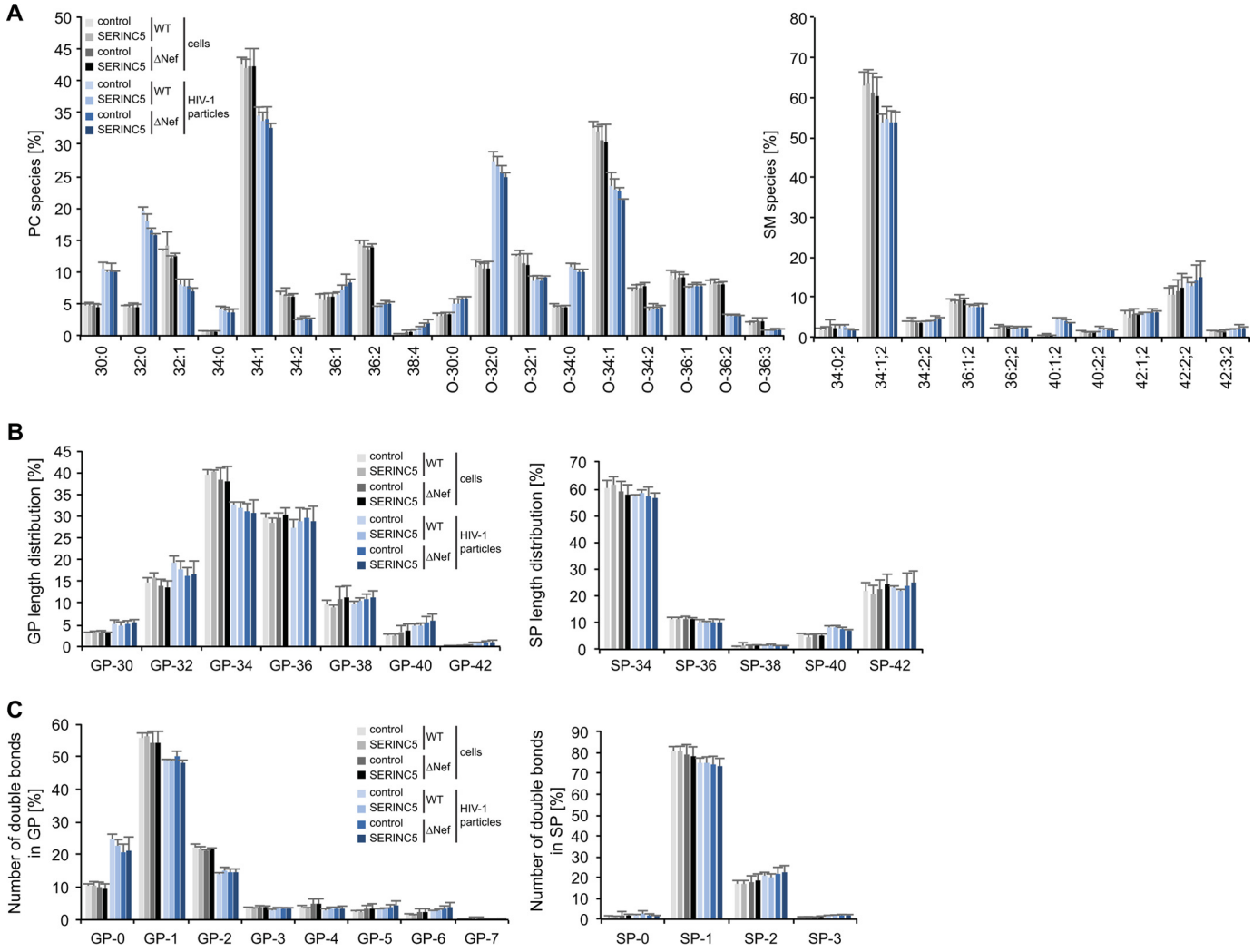


Figure 3. Lipid species compositions of HIV-1–producing cells and purified HIV-1 particles. Data are presented as mean ± S.D. of three independent experiments, except for ΔNef particles produced in the presence of SERINC5 ($n = 2$). *A*, molecular lipid species distributions of PC (*left panel*) and SM (*right panel*). PC species are shown as number of total carbon atoms:number of double bonds in fatty acyl residues. SM species are annotated with their number of carbon atoms, double bonds, and hydroxyl groups in the ceramide backbone, *e.g.* SM 34:2;1 contains in total in the sphingosine backbone and the fatty acyl moiety 34 carbon atoms, 2 double bonds, and 1 hydroxyl group. Species are standardized to 100% within each lipid class. *B* and *C*, distributions of chain lengths (*B*) and double bonds (*C*) within the categories GP (*left panels*) and SP (*right panels*). Species are standardized to 100% within each lipid category.

and antagonism by Nef occur in the absence of overall differences in lipid composition.

SERINC5 does not alter sphingosine metabolism

Our previous observation that expression of SERINC5 did not affect the global steady-state lipid composition of HIV particles did not exclude that the restriction factor alters the kinetics of lipid biosynthesis in functionally relevant ways. We therefore assessed next whether expression of SERINC5 alters the metabolism of sphingosine, the main building block of serine-dependent SP synthesis, which begins with the condensation of serine and CoA-activated palmitate, followed by the subsequent generation of sphinganine, ceramide, and key end products such as SM and glucosylceramide (Fig. 4*A*). To this end, we made use of a functionalized sphingosine (pacSph) containing a photoactivatable diazirine group and a clickable alkyne terminus to enable copper-catalyzed alkyne-azide cycloaddition. In cells, pacSph undergoes metabolism like its natural counterpart (50, 51), leading to the synthesis of complex functionalized sphingolipids. In the presence of sphingosine-1-phosphate lyase, an enzyme involved in degradation of sphingosine, the hydrocarbon backbone of pacSph, which contains the photoactivatable and clickable functionalities, is also fluxed into the glycerol- and phosphoglycerolipid biosynthetic pathway (50, 51) (Fig. 4*A*). Thus, the kinetics of SP, GP, and glycerolipids (GL) can be monitored at the same time. Cells cultured in the presence of pacSph were lysed after different times of incubation. Cell lysates were subjected to lipid extraction and copper-catalyzed alkyne-azide cycloaddition of a fluorescent dye (Fig. 4*B*). Lipids were separated by TLC to monitor the kinetics of SP, GP, and GL (Fig. 4*C*). Quantitative analysis of lipid classes showed that sphingosine metabolism in 293T cells were not affected by the presence of SERINC5.

SERINC5 restriction is not linked to altered phosphatidylserine exposure on HIV particles

As an alternative explanation for the effects of SERINC5 on HIV particle infectivity, we considered that the restriction fac-

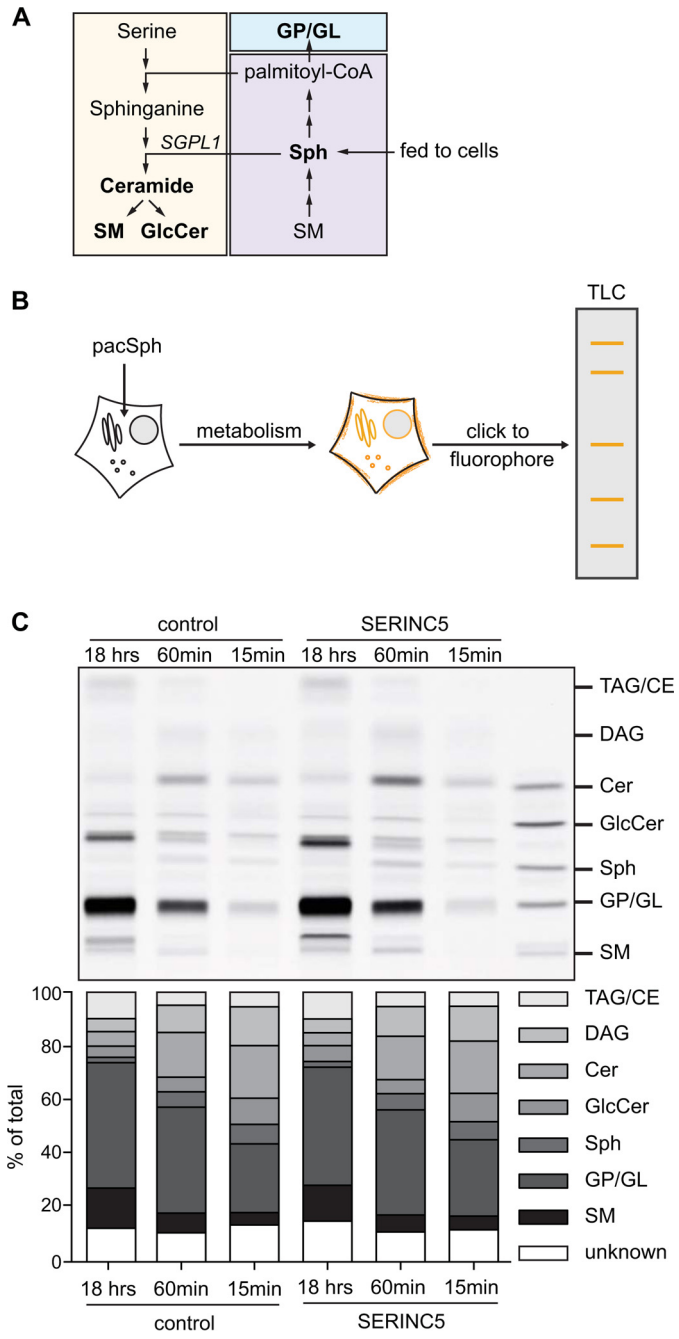
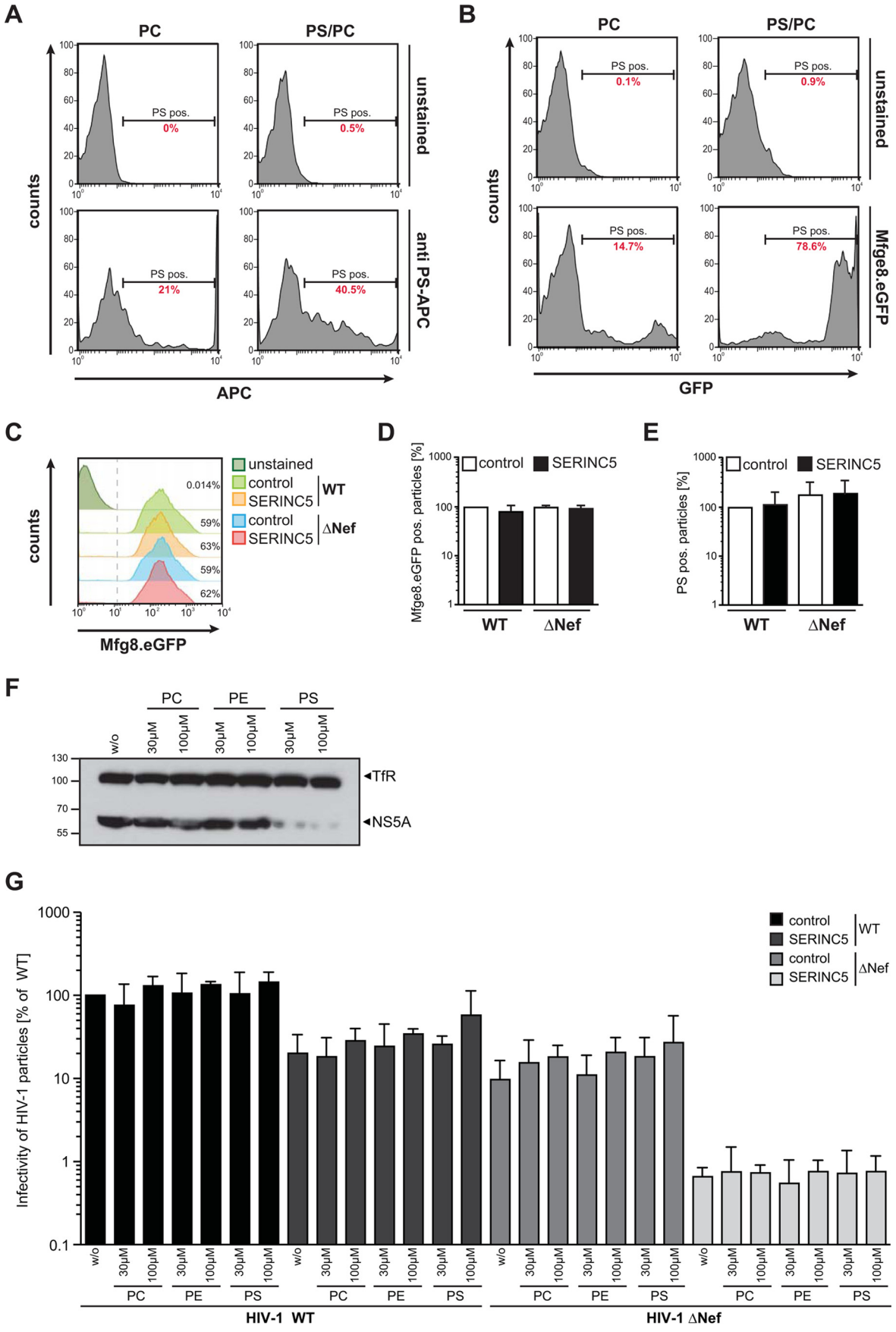


Figure 4. Analysis of sphingosine metabolism in the absence or presence of SERINC5. *A*, metabolism of sphingosine (*Sph*). pacSph fed to cells can either enter the biosynthetic (*orange*) or degradative (*purple*) pathway. In SP biosynthesis, ceramide serves as a branching point for the synthesis of phosphosphingolipids such as SM and glycosphingolipids such as glucosylceramide (*GlcCer*). Breakdown of sphingosine yield shuttles the hydrocarbon backbone via palmitoyl-CoA into biosynthesis of GL and GP. *B*, pacSph contains a photoactivatable diazirine group and a terminal alkyne moiety. pacSph is metabolized like its endogenous counterpart, entering both the biosynthetic and degradation pathways, giving rise to functionalized cellular lipids. Following lysis at different times of labeling, cells are subjected to a click reaction to couple a fluorescent reporter to alkyne-containing lipids. Lipid extracts were analyzed by TLC and fluorescence imaging to monitor Sph metabolism. *C*, cells were labeled with pacSph for 15 min, 60 min, or 18 h, lysed, and subjected to a click reaction using an azide-activated fluorophore. Following TLC separation, lipids were visualized by fluorescence imaging. Lipid classes were distinguished based on co-migrating standards, except for neutral lipids. Fluorescence signals were quantified, and relative contributions of lipid classes were calculated. *Cer*, ceramide; *DAG*, diacylglycerol; *GlcCer*, glucosylceramide; *TAG/CE*, triacylglycerol/cholesteryl ester.

tor may affect membrane organization rather than overall composition. In this context, the phospholipid PS was of particular interest. PS is generally present at the inner, cytoplasmic side of the plasma membrane of mammalian cells; however, specific triggers, such as the onset of apoptosis, lead to the translocation of PS to the outer leaflet of the plasma membrane and, thus, exposure at the cell surface (52). Physiologically, PS externalization serves as an uptake signal, *e.g.* for apoptotic cells by phagocytic cells. Such cells display specialized PS receptors, such as, *e.g.*, T cell immunoglobulin and mucin domain 1 (TIM) and Tyro-3, Axl, and Mer (TAM) proteins, at their surface that recognize membranes enriched in PS and whose engagement triggers the internalization pathways (53). Numerous viruses, including vesicular stomatitis virus, vaccinia virus, and hepatitis C virus (HCV), expose considerable amounts of PS on infectious particles, allowing them to hijack PS pathways for uptake and productive infection in a process referred to as apoptotic mimicry (54, 55). Although PS receptor-mediated uptake may lead to productive infection for viruses whose glycoproteins, *e.g.*, require activation by low pH, we reasoned that such entry pathways may be abortive for viruses such as HIV-1 that infect target cells in a mostly pH-independent manner. In this scenario, elevation of PS levels exposed at the surface of HIV-1 particles by SERINC5 could enhance the use of such abortive uptake and explain the observed restriction to particle infectivity.

To test this hypothesis, we first sought to quantify surface PS levels on HIV-1 particles and employed flow cytometry, following protocols for the analysis of liposomes of the size of HIV-1 particles (56) or of purified HIV-1 particles (57). As a positive control, liposomes with defined lipid composition and the approximate size of HIV particles (100–150 nm) were used. Liposomes only containing PC or a mixture of PC and PS (50% PS) were undistinguishable by size and granularity and did not display fluorescence when unstained (data not shown). Staining with a PS-specific antibody (anti-PS) (Fig. 5*A*) or with Mfge8.EGFP, a protein that binds to PS (58, 59) (Fig. 5*B*), readily detected PS-positive populations in PC/PS liposomes, and, in line with previous results, significantly reduced background binding was observed with PC liposomes (60). Using this setup, purified HIV particles were detectable by FACS and could be identified, *e.g.*, by the incorporation of Vpr.GFP (data not shown). We therefore stained purified HIV-1 WT and ΔNef particles produced in the absence or presence of SERINC5 with Mfge8.eGFP (Fig. 5, *C* and *D*). Surprisingly, a robust population of particles produced in the absence of SERINC5 was found to expose PS independently of Nef. The frequency and intensity of this PS positivity, however, were unchanged for virions produced from SERINC5-positive cells (Fig. 5, *C* and *D*). Although the percentage of HIV-1 particles detected as PS-positive varied between virions produced and stained on different days (60% for the particle preparations shown in Fig. 5*C*, ranging from 50–90%), virion preparations generated and analyzed in parallel displayed comparable PS positivity irrespective of expression of Nef or SERINC5. Comparable frequencies of PS-positive HIV-1 particles produced in the absence or presence of SERINC5 and/or Nef were also detected with the anti-PS antibody (Fig. 5*E*, 10–98% of HIV-1 particles detected as PS-positive). We conclude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C
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59%
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D
Mfge8.eGFP pos. particles [%]
E
PS pos. particles [%]
F
PE
PS
w/o
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100μM
130
100
70
55
TfR
NS5A
G
Infectivity of HIV-1 particles [% of WT]
HIV-1 WT
HIV-1 ΔNef

that HIV-1 particles carry a substantial amount of externalized PS independent of SERINC5 expression.

Competing with PS binding sites on target cells does not affect SERINC5 restriction

To test whether SERINC5 may introduce qualitative rather than quantitative changes to PS levels exposed on HIV particles, we next tested whether saturation of PS receptors on target cells affects SERINC5 restriction to virion infectivity. To this end, PC-, PE-, or PS-containing liposomes were incubated with target cells to block PS receptors and interfere with PS-dependent virus uptake. As a control, we used infection of Huh7 cells with HCV and scored the expression of the viral NS5A protein as a marker for productive infection by Western blotting. In line with a recent report (61), preincubation of Huh7 cells with PS but not PC or PE liposomes efficiently reduced HCV infection (Fig. 5*F*). In contrast, parallel treatment of TZM-bl cells had no effect on HIV-1 infection (Fig. 5*G*). In particular, the strong inhibition of HIV-1ΔNef infectivity by SERINC5 was maintained even in the presence of 100 μM PS receptor-competing PS liposomes. Together, these results illustrate that restriction of HIV-1 infectivity by SERINC5 does not involve targeting of HIV-1 particles to non-productive apoptotic mimicry entry routes.

Discussion

Based on the reported ability of SERINC proteins to feed serine into SP biosynthesis pathways, an attractive and often suggested model predicts that its pronounced negative effect on virus infectivity reflects alterations in virion lipid composition (7, 8, 34, 38, 62). The goal of this study was to test this hypothesis and assess whether expression of SERINC5 alters the lipid composition and organization of HIV-1 particles and whether such putative effects contribute to the antiviral activity of SERINC5. Our results do not support these hypotheses because, under experimental conditions where infectivity of HIV-1ΔNef was potently suppressed, expression of SERINC5 during HIV production did neither affect sphingosine metabolism of producer cells nor the overall lipid composition or PS externalization of HIV particles.

Together, these results seem to refute the idea that the restriction of SERINC5 to HIV infectivity is mediated by alterations in HIV lipid composition. Although our quantitative lipidomics analysis covered a comprehensive range of all major lipid classes, these analyses cannot exclude the involvement of rare lipid species we were not able to detect by our mass spectrometry approach. However, it seems unlikely that a serine incorporation function of SERINC5 would be required for such an effect because we did not observe any difference in the abundance of any of the key products of serine-dependent lipid biosynthesis that would also be required for the generation of such still to be determined lipids. Our bulk analysis of whole cell membrane lipids also does not exclude that SERINC5 may have subtle effects on the lipid composition of the plasma membrane, from which HIV particles bud. Such changes, however, should become apparent in virions whose lipid composition was unaffected by SERINC5 in our analysis. We therefore favor the idea that SERINC5 suppresses HIV virion infectivity by alternative mechanisms. In line with a previous report on the effect of Nef on the accessibility of Env in virions to antibody neutralization (63), such effects could include alterations in organization and abundance of Env-containing microdomains at the plasma membrane and in virions. In this scenario, SERINC5 may create a local microenvironment that impairs the fusogenic potential of Env by physical association or indirect mechanisms (38). Such effects of SERINC5 may well be independent of its putative serine incorporator activity. Alternatively, serine incorporation may allow SERINC5 to affect metabolic processes other than lipid biosynthesis that impact HIV particle infectivity.

In addition to the mechanism of antiviral activity of SERINC5, the findings presented here expand our knowledge of the general properties of this restriction factor. The name-giving study by Inuzuka *et al.* (35) characterized SERINC proteins as transmembrane proteins that have the ability to associate with key enzymes of SP biosynthesis and to incorporate serine into membranes, which suggests that they may facilitate the synthesis of PS and SP. Our comparison of SERINC5-negative 293T cells with 293T cells that overexpress functional levels of SERINC5 did not reveal significant differences in abundance of SP and PS. Serine incorporation by SERINC5 therefore does not seem to contribute to the biosynthesis of these lipids in this experimental system. Similar findings were made recently in a mouse strain in which expression of SERINC1 is abolished by retroviral insertion but did not affect the composition of serine-derived lipids in lymphocytes and macrophages (64). The fact that no significant changes in cellular SP composition were observed upon manipulation of individual SERINC proteins may reflect compensation of the serine incorporator function by other members of the SERINC pro-

Figure 5. PS surface levels on liposomes and HIV-1 particles and liposome competition for PS binding sites in the context of HIV-1 infection. *A*, percentages of PS-positive liposomes detected with PS-specific antibody. PC- and PS/PC-containing liposomes (100 μl of 1 mM liposome stock solution) were incubated with a PS-specific antibody (1:100, 1 h at 4 °C) followed by an anti-mouse APC-coupled secondary antibody (1:200, 1 h, 4 °C) and analyzed by flow cytometry. Unstained liposomes served as a control. *B*, percentages of PS-positive liposomes detected with Mfge8.eGFP. PC- and PS-containing liposomes (100 μl of 1 mM liposome stock solution) were incubated with recombinant Mfge8.eGFP (1:100) and analyzed via flow cytometry. Unstained liposomes served as a control. *C*, percentages of PS-positive HIV-1 particles stained with recombinant Mfge8.eGFP. HIV-1_{NL43} WT or ΔNef particles (50 μl with approximately 5×10^{10} picounits RT/μl) produced in the absence (control) or presence of SERINC5 (*SERINC5*) were incubated with Mfge8.eGFP (1:100) and analyzed by flow cytometry. Unstained virus particles served as a control. *D*, quantification of PS-positive HIV-1 particles as analyzed in *C*. Percentages of PS-positive particles as determined by the Mfge8.eGFP signal are shown relative to HIV-1 WT particles + control (set to 100%) ($n = 3$). *E*, quantification of PS-positive HIV-1 particles analyzed with an anti-PS antibody. Percentages of PS-positive particles (average values from four independent experiments) are shown relative to HIV-1 WT particles + control (set to 100%). *F*, Western blot analysis of Huh7.5 cells infected with HCV (multiplicity of infection = 10) at 37 °C for 8 h in the absence (*w/o*) or presence of different concentrations (10 and 30 μM) of PC-, PE-, or PS-containing liposomes. Cells were lysed 48 h after infection and subjected to SDS-PAGE, and HCV NS5A was detected via immunostaining. TfR served as a loading control. Shown is one representative of two independent experiments. *G*, HIV-1 infection of TZM-bl cells in the absence or presence of different concentrations (10 and 30 μM) of PC-, PE-, or PS-containing liposomes. HIV-1 WT or ΔNef (2×10^8 picounits RT/μl) produced in the absence (control) or presence of SERINC5 (*SERINC5*) was used for the infection. 48 h after infection, cells were lysed, and infection rates were determined via luciferase assay. Infection rates are presented relative to HIV-1 WT + control, which was arbitrarily set to 100%. Shown are means of two independent experiments, each performed in triplicate.

tein family. Cell system–specific differences in the relative contribution of individual SERINC proteins to SP synthesis may also exist. Nevertheless, it needs to be considered that the serine incorporator activity demonstrated by Inuzuka *et al.* (35) does not primarily feed into SP synthesis or may not even represent the key physiological activity of SERINC proteins.

Together, the results presented here demonstrate that the potent antiviral activity of SERINC5 does not rely on alterations of host cell and virion lipid composition and warrant further investigation into the biological activities of SERINC proteins.

Experimental procedures

Cell lines, reagents, and plasmids

293T cells were cultivated in DMEM with 10% FCS and 1% penicillin-streptomycin (all from Invitrogen). The following antibodies were used: mouse anti-HA.11 (clone 16B12, Biolegend), mouse anti-phosphatidylserine (clone1H6), sheep anti-HIV-1 capsid (p24) antiserum (kindly provided by Barbara Müller), sheep anti-Nef (arp444, National Institutes of Health AIDS repository), mouse anti-NS5A (9E10, kindly provided by Charles Rice), mouse anti-transferrin receptor/CD71 clone H68.4 (Thermo Fisher), and secondary allophycocyanin (APC)-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories). Human Mfge8.eGFP fusion protein, a fusion protein of full-length human milk fat globule epidermal growth factor 8 (Mfge8), also known also lactadherin, was produced from stably transfected HEK293 cells. Mfge8.eGFP binds to PS with high affinity, but not PE, PI, or PG, and displays reduced affinity to PS (60). HEK293 cells were grown in a Labfors Bioreactor (Infors) in serum-free medium (Ex-Cell 293, Sigma) for 5 days. Then cells were removed from the cell culture supernatant by centrifugation (300 × *g*, 10 min). Supernatant was cleared from debris by a high-speed centrifugation (20,000 × *g*, 90 min) and filtration (0.2 μm). Mfge8.eGFP was then purified by FLAG affinity chromatography using M2-FLAG-agarose beads (Sigma). Bound protein was eluted using an excess of FLAG-peptide (Genscript). Finally, the eluate was concentrated using Amicon spin columns (Amicon Ultra-15, Millipore).

The following DNA constructs were used. The plasmids for the expression of GFP fusion proteins of SERINC5 were described before (7). pBJ5-based constructs for the expression of internally HA-tagged SERINC5 (pBJ5_SERINC5.intHA or empty control vector) were a kind gift from Heinrich Göttlinger (8). The proviral chimera pHIV-1_{NL4-3} SF2 Nef WT (HIV-1 WT) was described previously (65). The proviral plasmid pHIV-1_{NL4-3} Nef stop (ΔNef) was kindly provided by Frank Kirchhoff. The plasmid pFK-J6/C3(Jc1 WT) (66) was used for *in vitro* transcription and HCV stock production.

Virus production and purification of HIV-1 particles

Virus was generated by transfection (via calcium phosphate) of proviral HIV-1_{NL43} plasmids into HEK293T cells. For this, 5×10^6 293T cells were seeded per 150-cm^2 flasks 1 day before co-transfection of proviral DNA (70 μg) and plasmid DNA for expression of internally HA-tagged SERINC5 (7 μg) or a vector control. HIV-1 particles were purified as described previously (39, 41). Briefly, 2 days after transfection, culture supernatants were harvested, cleared, and concentrated through a 20% (w/v) cushion of sucrose in PBS. Concentrated virus was further purified via velocity gradient centrifugation on an OptiPrep gradient (Axis-Shield, Oslo, Norway). The visible virus band was collected and pelleted (44.000 rpm, 45 min). The amount of released, purified viral particles was determined using a one-step SYBR Green I—based, product-enhanced reverse transcriptase assay (67). For that, 5 μl of virus-containing supernatant was lysed in 2× virus lysis buffer for 10 min and diluted with 90 μl of PCR buffer, and 10 μl thereof was used for the RT-PCR reaction. Absolute values of the virus suspensions tested were retrieved from a standard curve created using serial dilutions of a previously characterized virus stock. The relative infectivity of purified virus particles was determined by infection of TZM-bl reporter cells cultured in 96-well format. Infectivity was determined 48 h after infection by analysis of firefly luciferase activity (measured in relative light units) as described previously (68). The purity of the virus particle preparation was determined via silver staining of SDS-polyacrylamide gels. For Western blot analysis of virions, virus particle preparations were lysed in 2–6× SDS sample buffer containing 50 mM tris(2-carboxyethyl)phosphine (0.5 M stock (pH 7.0), Sigma).

Single-cell suspensions of human hepatoma-derived Huh7.5 cells were prepared by trypsinization and washed once with PBS. Cells were resuspended at a concentration of 1×10^7 cells/ml in Cytomix (69) containing 2 mM ATP and 5 mM glutathione. 10 μg of *in vitro* transcribed RNA (70) was mixed with 400 μl of the cell suspension and transfected by electroporation with a Gene Pulser system (Bio-Rad) in a cuvette with a gap width of 0.4 cm (Bio-Rad) at 975 microfarad and 270 V. Cells of two transfections were immediately transferred into medium and seeded into a 15-cm-diameter culture dish. Supernatants were collected 48 h after electroporation and filtered through a 0.45-μm-pore membrane, and virus particles were concentrated by ultrafiltration using a centrifugal filter device (Centricon Plus-70, Millipore). Concentrated culture supernatant was stored at −70 °C. Infectivity titers of virus stocks were determined by limiting dilution assay using Huh7.5 cells as described elsewhere (71).

Western blotting

Cells were lysed in 2× SDS sample buffer containing 50 mM Tris(2-carboxyethyl)phosphine (0.5 M stock in H_2O (pH 7.0), Sigma), lysates were sonicated, and proteins were separated on 12.5% SDS gels and blotted to nitrocellulose membranes. Membranes were blocked in 5% milk in phosphate buffered saline Tween-20 (PBS-T) and probed with the following primary antibodies: mouse anti-HA (1:1000), sheep anti-HIV-1 p24CA antiserum (1:5000), sheep anti-HIV-1 Nef (1:1000), mouse anti-TfR (1:500), and mouse anti-HCV NS5A (1:20000). Secondary antibodies were conjugated to horseradish peroxidase for ECL-based detection.

Quantitative lipid analysis

Cells and viral particles were subjected to acidic Bligh and Dyer lipid extractions (72). Lipid standards were added prior to extractions using a master mix containing phosphatidylcholine (13:0/13:0, 14:0/14:0, 20:0/20:0; 21:0/21:0; Avanti Polar Lipids) and sphingomyelin (d18:1 with *N*-acylated 15:0, 17:0, 25:0;

semisynthesized as described in Ref. 43), D6-cholesterol (Cambridge Isotope Laboratory), phosphatidylinositol (16:0/16:0, 17:0/20:4; Avanti Polar Lipids), phosphatidylethanolamine and phosphatidylserine (both 14:1/14:1, 20:1/20:1, 22:1/22:1; semisynthesized as described in Ref. 43), ceramide and glucosylceramide (both d18:1 with *N*-acylated 15:0, 17:0, 25:0; semisynthesized as described in Ref. 43), phosphatidic acid (17:0/20:4, Avanti Polar Lipids), and phosphatidylglycerol (14:1/14:1, 20:1/20:1, 22:1/22:1; semi-synthesized as described in Ref. 43). Evaporated lipids were redissolved in 60 μl of 10 mM ammonium acetate in methanol and analyzed on a QTRAP6500+ mass spectrometer (Sciex) with chip-based (HD-D ESI Chip, Advion Biosciences) electrospray infusion and ionization via a Triversa Nanomate (Advion Biosciences) as described previously (43). Redissolved lipid extracts were diluted 1:10 in 96-well plates (Eppendorf Twintec 96, colorless, Sigma, Z651400-25A) prior to measurement. Precursor and neutral loss scanning in positive ion mode was employed to measure glycerophospholipids as described previously (43). The remaining samples were subjected to cholesterol determination as described previously (73). Data evaluation was done using LipidView (ABSciex) and a software developed in-house (ShinyLipids).

Analysis of sphingosine metabolism

3×10^5 293T cells were seeded in 6-well plates and transfected with 2.5 μg of GFP or SERINC5.GFP expression plasmids for 24 h. For pacSph labeling, medium was removed, and cells were washed with PBS and labeled with DMEM containing 10% delipidated FCS and 2 μM pacSph for 18 h, 60 min, 15 min, or 0 min. Labeling medium was removed, and cells were detached using cell dissociation buffer (Thermo Fisher, 13151014), transferred into 1.5-ml tubes, and pelleted (300 $\times$ *g*, 5 min, 4 °C). Cell pellets were washed three times with PBS and resuspended in 300 μl of PBS. For lipid extractions, 600 μl of MeOH and 150 μl of $CHCl_3$ were added, and samples were mixed by vortexing. Proteins were pelleted (14,000 $\times$ *g*, 5 min), and supernatants were transferred into 2-ml tubes. Following addition of 300 μl of $CHCl_3$ and 600 μl of 0.1% acetic acid in H_2O, samples were vortexed and centrifuged (14,000 $\times$ *g*, 5 min). The lower organic phase was transferred into a 1.5-ml tube, and the solvent was evaporated in a SpeedVac at 30 °C. Lipids were redissolved in 7 μl of $CHCl_3$ and 30 μl of freshly prepared click mixture containing 3 μl of 44.5 mM 3-azido-7-hydroxycoumarin (Jena Bioscience, CLK-FA047-1) in DMSO, 250 μl of 10 mM Tetrakis(acetonitrile)copper(I) tetrafluoroborate (Sigma-Aldrich, 677892) in MeCN, and 1 ml of EtOH. Click reactions were performed in a SpeedVac at 45 °C for 20 min. Samples were dissolved in 20 μl of $CHCl_3$:MeOH:H_2O:AcOH (65:25:4:1, mobile phase 1) and spotted on a HPTLC silica gel 60 plate (Merck, 1.05641.0001) using a CAMAG Linomat 5 applicator. HPTLC plates were developed to 60% with mobile phase 1 and to 100% in mobile phase 2 (hexane:ethyl acetate, 1:1) in a CAMAG ADC2 system. For enhanced fluorescence of the coumarin derivative, the plates were sprayed with 4% (v/v) Hünig's base (Sigma-Aldrich, 387649) in hexane. HPTLCs were imaged with the Amersham Biosciences Imager 600 at 460 nm.

Generation of liposomes

Lipid stocks solutions of DOPC, DOPE and DOPS (Avanti Polar Lipids, 10 mg/ml in $CHCl_3$) were prepared. Lipids were mixed and dried under nitrogen and a vacuum for 1 h. Lipids were solubilized in PBS to obtain a final concentration of 1 mM. Liposomes were prepared by 10 freeze-thaw cycles of warming to 42 °C and freezing in liquid nitrogen (−196 °C) and sized by extrusion through a 50-nm polycarbonate filter using a mini extruder device (Avanti Polar Lipids). Size distributions of liposomes were measured by dynamic light scattering (Wyatt Technology).

FACS analysis of HIV particles or liposomes

Staining of PS on liposomes (100 μl; total lipid concentration, 1 mM) or HIV particles (50 μl of 5×10^{10}-1×10^{11} picounits RT/μl) was performed by staining with a mouse anti-PS antibody (1:100 in PBS) or Mfge8.eGFP (1:100 in PBS) for 1 h at 4 °C. Liposomes treated with anti-PS antibody were stained with an APC-coupled anti-mouse secondary antibody (1:200). The liposome antibody or Mfge8.eGFP mixture was filled up to 1 ml with PBS to wash out unbound antibody or Mfge8.eGFP. Liposomes and virus particles, respectively, were pelleted through a cushion of 20% sucrose in PBS (44.000 rpm for 45 min) and resuspended in 300 μl of PBS. Stained HIV-1 particles were inactivated with paraformaldehyde (3% final concentration) for at least 90 min at room temperature. HIV-1 particles and liposomes were analyzed with a FACS Calibur instrument (BD Biosciences), and data were processed with FlowJo software.

Liposome competition experiments

For HCV infections in the presence of liposomes, Huh-7.5 cells were incubated with 10 or 30 μM liposomes or medium as control for 30 min at room temperature and then infected with HCV Jc1 at a multiplicity of infection of 10 in the presence of liposomes (10 or 30 μM) at 37 °C for at least 8 h. 48 h after infection, cell lysates were harvested for detection of HCV NS5A by Western blot analysis using an NS5A-specific monoclonal antibody. Transferrin receptor (TfR) was used as a loading control. For HIV-1 infections in the presence of liposomes, TZM-bl cells were incubated with 10 or 30 μM liposomes or medium as control for 30 min at room temperature and then infected with HIV-1 (2×10^8 picounits RT/μl) for 6 h. 48 h after infection, cells were lysed, and the infectivity of HIV-1 particles was determined by analysis of firefly luciferase activity.

Statistical analysis

Statistical analysis of datasets was carried out using Microsoft Excel and GraphPad Prism. Statistical significance of parametrically datasets was analyzed by unpaired two-tailed Student *t* test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Author contributions—O. T. F. and B. B. designed the study and wrote the manuscript. B. T. and B. G. purified HIV-1 particles. C. L. and B. B. performed experiments for quantitative lipid analysis. B. T. performed and analyzed the experiments shown in Figs. 1–3 and 5 and contributed to the preparation of all figures. V. P. and H. W. performed and analyzed the experiments shown in Fig. 4. J. K., T. B., M. P., H. G. K., and A. R. provided reagents and expertise. All authors approved the final manuscript.

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