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Letter

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Lipid Composition of the Viral Envelope of Three Strains of Influenza Virus—Not All Viruses Are Created Equal

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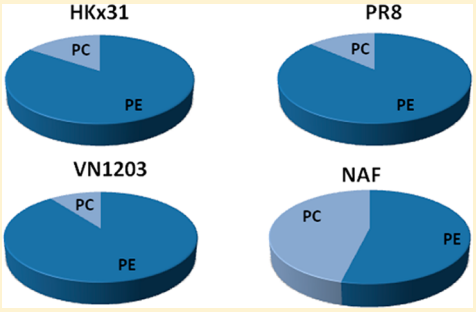
Supporting Information

ABSTRACT: Although differences in the rate of virus fusion and budding from the host cell membrane have been correlated with pathogenicity, no systematic study of the contribution of differences in viral envelope composition has previously been attempted. Using rigorous virus purification, marked differences between virions and host were observed. Over 125 phospholipid species have been quantitated for three strains of influenza (HKx31-H3N2, PR8-H1N1, and VN1203-H5N1) grown in eggs. The glycerophospholipid composition of purified virions differs from that of the host or that of typical mammalian cells. Phosphatidylcholine is the major component in most mammalian cell membranes, whereas in purified virions phosphatidylethanolamine dominates. Due to its effects on membrane curvature, it is likely that the variations in its content are important to viral processing during infection. This integrated method of virion isolation with systematic analysis of glycerophospholipids provides a tool for the assessment of species-specific biomarkers of viral pathogenicity.



KEYWORDS: *lipidomics, virion, influenza, phospholipids, plasmalogens, membrane lipid composition, infectious disease*

Viruses hijack the enzymatic machinery of host cells to generate the necessary building blocks for new virus replication. Whereas the glycerophospholipids (GPLs) in mammalian cells are highly organized and their composition is tightly regulated, pathogenic organisms and viruses can tolerate large variations in the GPL composition of their envelopes.[1] Previous work from our group has shown that the GPL composition of the infected systems may be only subtly modified from that of noninfected cells and that the purified virion lipid composition is drastically different from that of either.[2] Influenza viruses of different pathogenicity have been associated with high mortality and development of severe complications.[3] Influenza A virus is an enveloped, negative-sense, single-stranded RNA virus that can acquire mutations, and some can be resistant to the currently available antiviral drugs.[4] Viral envelopes possess relatively strong positive curvature owing to the inert extracellular lipid bilayers of their membrane. Variations in lipid composition of the viral envelope can be correlated to virus infectivity as they modulate viral fusion.[5] Lipids such as free fatty acids or lysophosphatidic acid (inverted cone lipids) are known to promote positive curvature by introducing inverted cone shape into the membrane, whereas cone-shaped lipids such as phosphatidylethanolamine, lysolipids, or phosphatidic acid tend to adopt the negative curvature required for fusion.[6,7]

As a basis for evaluating differences in the lipid composition that may affect viral pathogenicity, three influenza viruses were studied here: PR8/H1N1,[8] a highly pathogenic strain in laboratory mice, which for the past 30 years has been used to produce inactivated influenza vaccine; X31/H3N2,[9,10] a low-pathogenicity strain; and a modified VN1203/H5N1,[11] lethal to domestic chickens and highly pathogenic in ferret and mouse models. All viruses in this study were grown in eggs. The three viruses used were all generated by reverse genetics on the PR8 backbone to compare differences in virion composition associated with differences in the surface hemagglutinin (HA) and neuraminidase (NA) proteins. The choice for a growing medium was based on this well-characterized system used by CDC for vaccine development and production. Over 125 phospholipid species have been quantitated in each strain and in the host—noninfected egg allantoic fluid. The quantitative analysis of low-abundance lipid species led to the identification of subtle differences between strains, which may lead to a potential correlation between phospholipid composition and influenza severity.

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Alteration of the cellular membrane plays an important role in viral replication and infectivity.[12] The viral lipid membrane is somewhat similar to that of the host cells as they rely on the host for membrane structural fragments, but it also involves de novo lipid synthesis.[13] Evaluation of the structural lipid composition of the viral envelope is essential when strains of different lethality or morbidity are compared. One of the major prerequisites for an accurate determination of the viral lipid composition is the virion purification and normalization between samples. This allows assurance that the amount of lipid recovered from an individual preparation reflects similar infection/growth trajectories and degree of purification as compared to another replicate experiment. The procedure utilized in this study reliably produced pure virion particles suitable for further analysis (Figure 1). Interest in the lipid composition of the viral envelope

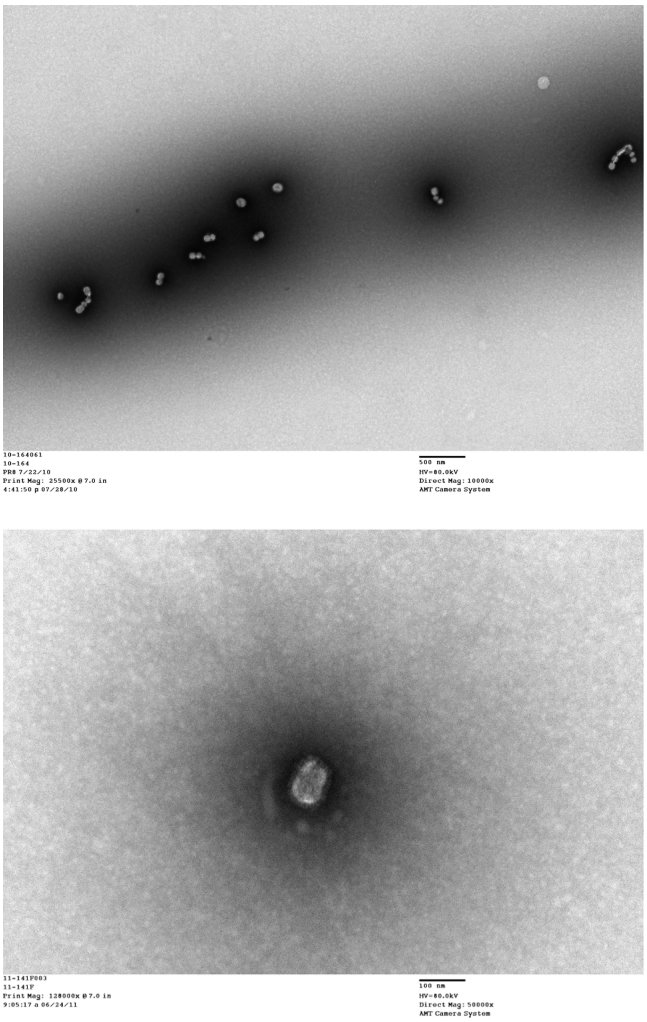

Figure 1. Representative electron micrograph of purified PR8, grown in egg.

and its relation/comparison to that of its host dates several decades back and provides an excellent foundation for this study.[14−16] The advancement in lipidomics technology took this exploration further and allowed a more detailed interrogation of that system at the molecular species level. Profiling of the purified virions and noninfected allantoic fluid (NAF) allowed a comprehensive characterization and comparison of the major phospholipid classes' composition at a molecular species level between all preparations. Absolute quantitation of over 125 molecular species in each strain and host was achieved by the use of LC-MS analysis and computational platforms developed in our laboratory.[17] In all, more than 200 molecular species (including isobaric species) were identified in the seven major classes—phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) as well as their respective lysolipids in each strain and NAF (Supplementary Tables S1−S4 in the Supporting Information). This is an illustration of the large number of lipid species present in virions. Whereas some of the classes are remarkably similar in number of species present and their composition between strains (e.g., PG, PA, SM), others show a marked difference in diversity and quantity (PC, PE, PS).

The glycerophospholipid makeup of the purified virions is different from that of the typical mammalian cells.[2] PC is the major structural component of the mammalian cell membrane, comprising about 50% of the total lipids, and the second largest fraction is PE. In purified virions PE is the major class of the phospholipids. The ratio of PE to PC in NAF is close to 1, whereas in purified virions it is between 5 and 9 (Figure 2a). In addition to the large differences between the lipidome of the virions as compared to the NAF, there were also differences in the lipid composition between strains. The diversity in the GPL composition between the three strains and NAF is presented in Figure 2 for major diacyl phospholipid classes and SM and in Table 1.

As shown in Table 1a, NAF exhibits a GPL profile similar to that of most mammalian systems and corresponding well with published data.[18] At the same time virions grown in egg reveal much lower amounts of the structural phospholipid PC and the anionic phospholipids PA, PG, and PI, whereas PE and PS are present to a higher extent. Considering the role of the spatial phospholipid presentation and charge and their role in membrane dynamics, this class distribution clearly demonstrates that virions require a membrane configuration favorable for fusion, which is dependent on lipid composition.[19] Influenza viruses also contain higher sphingomyelin content than their host cells, and this phenomenon is associated with the ability of the virus to infect cells.[20] It is very likely that this rise is an illustration of the expansion in lipid rafts in the virion lipid membrane compared to that of NAF. Influenza viruses have been shown to use raft platforms for budding.[21,22] Thus, enrichment in SM provides lipid raft organization, which not only influences membrane fluidity but also facilitates virus budding.[23] The three viral strains studied here also differ in their GPL composition. HKx31 displays the most structural lipids (cylindrical steric presentation) (PC, PI, PG) compared to other strains, whereas VN1203 has the least. At the same time PE content of VN1203 is the largest, and this strain also shows the highest PE:PC ratio of 9. Cone-shaped lipids such as PE with suitable molecular geometry facilitate appropriate membrane fluidity and dynamics necessary for virus infection.[7,24] Modification in the PE content can probably be exploited as a means to decrease its content and thus render viruses unable to acquire the necessary membrane conformation by affecting the de novo PE biosynthesis via ethanolamine kinase inhibition.[25] Another phospholipid class, which just like PE is predominantly found in the inner membrane leaflet, PS, appears to enhance virus entry through interaction with annexin proteins[26,27] and has also been implicated in augmented infectivity and viral transmission of enteroviruses.[28] All three strains show similar PS contents, much higher than in the NAF host. Apart from lysophosphatidic acid (LPA),

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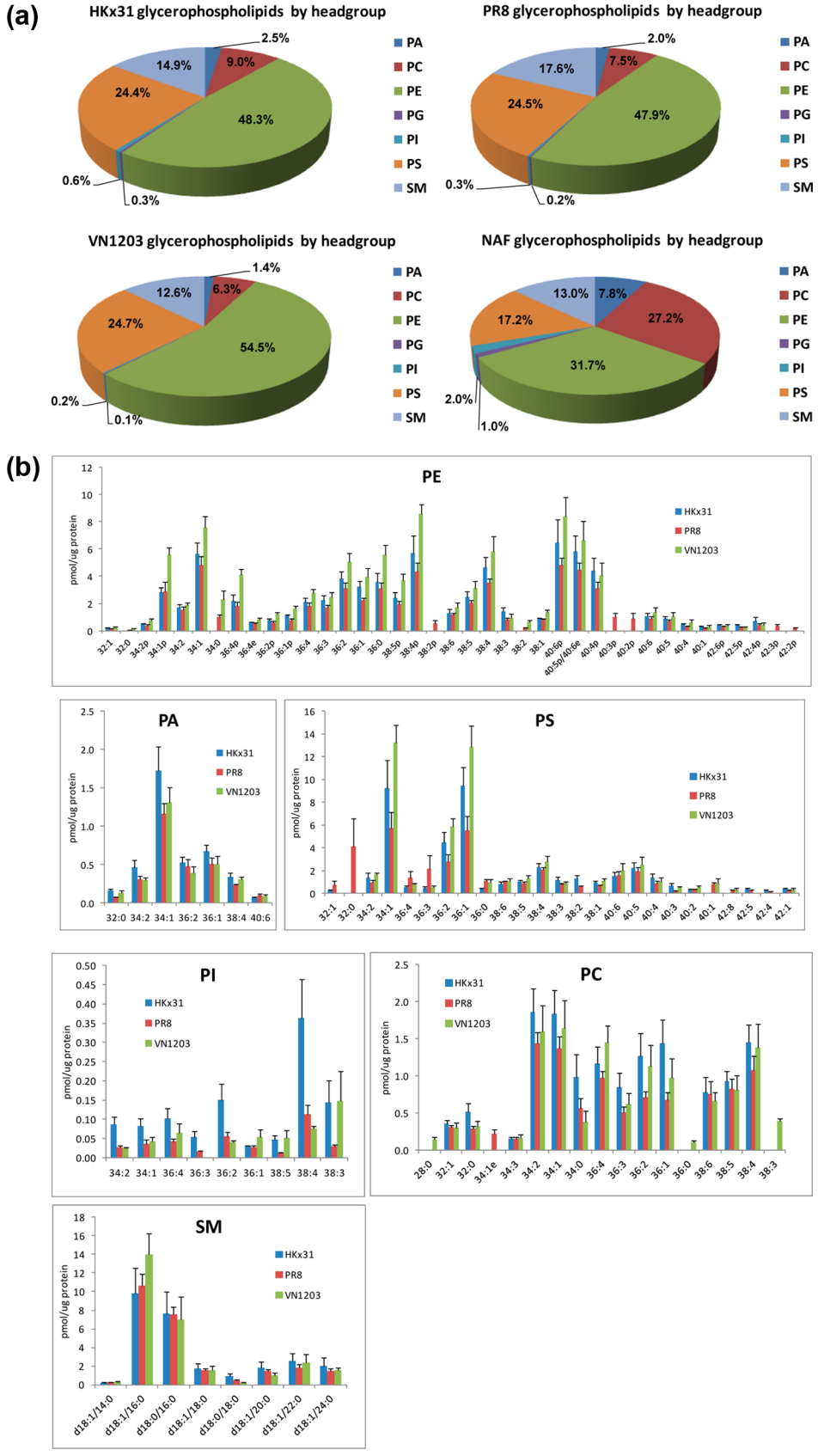

Figure 2. (a) GPL profiles of egg-grown influenza virions differ from each other and that of noninfected allantoic fluid (NAF). (b) Species level bar graphs in major diacyl phospholipid classes and sphingomyelin.

lysolipids were essentially not detected in the NAF, but there was considerably more LPA detected in the NAF than the virions (Table 1b). Additionally, the molecular fraction of this class of lipid species was significantly elevated in PR8 relative to the other

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Table 1. Comparison of Major GPL Class Distribution between Three Influenza Strains and Host (NAF) and Lysolipids in Each Influenza Strain and NAF

	HKx31 (%, n = 8)		PR8 (%, n = 8)		VN1203 (%, n = 8)		ANOVA p value	NAF (%, n = 9)	
	mean	sem	mean	sem	mean	sem	across virions	mean	sem
(a) headgroup									
%PA	2.5	0.3	2.0	0.3	1.4	0.1	0.014	7.8	0.5
%PC	9.0	0.7	7.5	0.9	6.3	0.5	0.042	27.2	2.19
%PE	48.3	2.1	47.9	2.4	54.5	1.0	0.042	31.7	1.0
%PG	0.3	0.0	0.2	0.0	0.1	0.0	0.002	1.0	0.1
%PI	0.6	0.1	0.3	0.1	0.2	0.0	0.007	2.0	0.2
%PS	24.4	0.8	24.5	0.4	24.7	0.4	0.929	17.2	0.9
%SM	14.9	1.4	17.6	1.2	12.6	1.0	0.027	13.0	0.6
%PEp of total GPL	19.5	1.0	18.9	0.7	22.4	1.0	0.031	17.2	1.0
PE/PC ratio	5.3	0.6	7.0	1.2	9.0	0.8	0.035	1.2	0.1
(b) lysophospholid class									
%LPA	0.03	0.01	0.12	0.05	0.03	0.02	0.042	3.8	0.4
%LPC	0.87	0.12	0.80	0.10	1.08	0.07	0.140		
%LPE	7.5	0.6	8.7	1.3	10.3	1.0	0.162		
%LPG	0.02	0.00	0.02	0.00	0.03	0.01	0.553		
%LPI	0.04	0.01	0.01	0.01	0.22	0.03	0.190		
%LPS	1.0	0.2	1.0	0.1	0.9	0.1	0.321		
%lyso of total GPL	9.5	0.8	10.5	1.3	12.3	1.0	0.172	3.8	0.4

two virus strains (p = 0.04). Lysophosphatidylethanolamine (LPE) was the largest lysolipid class represented in the virions. The VN1203 strain had the highest molecular fraction (10.3%) of this class. A difference in the overall lysolipids as a proportion of the total GPL pool was also observed, with VN1203 displaying the highest at 12.3%, followed by PR8 with 10.5% and HKx31 with 9.5%, whereas there was only 3.8% of lysolipid quantitated in NAF (Table 1b). Incorporation of lysolipids in the membrane leads to increased permeability,[29] which can be beneficial for cytosolic constituent exchange during viral replication and assembly. The differences in lysolipid distribution lead to different membrane profiles and can probably be implicated in different viral infectivities.[7,29]

The fatty acid composition of each class of GPLs was examined. HKx31 was the most different from the other two virions, with noticeably more representation by polyunsaturated (PUFA) PS and PI species and less PUFA PG (Figure 3a,b). The NAF contains very little PUFA outside PC, PI, and PS. NAF displayed a different profile of fatty acid unsaturation across classes compared to the virions, particularly for PA and PG, where no PUFA species were above the limit of quantitation in NAF. Similar relationships exist when the data are viewed by the percentage of long chains (>36 carbons) in each class (Supplementary Figure S1). Comparison of the fatty acid (FA) composition between the viral strains and that of the NAF demonstrated some differences in the saturated fatty acid-containing phospholipids. As an illustration, extracts of VN1203 and PR8 virions had higher fractions of saturated FA-containing LPE, PE, and LPC compared to HKx31 and NAF (Table 2 and Supplementary Table S5). The elevated presence of phospholipids containing the "initiators" of the lipid biosynthesis (palmitic, C16:0; and stearic acids, C18:0) is an indication of lipogenesis. Similar up-regulation of de novo lipid biosynthesis has been previously reported during DENV infection.[30]

Viral budding and entry depend on the disruption of the original membrane bilayers and the generation of a spontaneous membrane curvature, which would allow membrane rupture and complete fission or fusion actions. Accumulation of curvature-forming lipids (cone or inverted cone) and decrease of cylindrical lipids would result in a highly curved membrane.[24] Viral envelopes of HCMV[2] or influenza virus[18] showed augmented PE content. Recent lipidomics analysis of HCMV and HIV demonstrated strong enrichment of the virion in plasmalogen ethanolamines (PEp)[2,31] a highly fusogenic lipid,[32] as a fraction of total GPLs. Plasmalogen species have different physical properties from their diacyl counterparts and are well-known to affect the membrane fluidity.[32] Several studies suggest viruses bud from lipid raft formations, requiring viral lipid membrane modification.[21,23,33] Increased SM content in purified virions compared to that of NAF is consistent with a rise in lipid raft domains and their utilization for viral replication, membrane fusion, intracellular transport, etc. Viruses benefit from sphingolipid metabolism and alter cellular signaling, as with the influenza A virus, which activates sphingosine kinase 1 and the transcription factor NF-κB.[22]

METHODS

Materials. All glycerophospholipids used as standards in the MS analysis were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). HPLC grade solvents were supplied by VWR (West Chester, PA, USA) and used without further purification.

Virion Cultivation and Purification. The H1N1 virus studied was a reverse genetics-generated A/Puerto Rico/8/1934 (PR8). The H3N2 and H5N1 viruses were recombinant strains made on the PR8 backbone: A/Aichi/2/1968xPR8 (HKx31) and A/Vietnam/1203/2004 (VN1203). The VN1203 strain has a modification in the HA protein to remove the polybasic cleavage site, allowing it to be used under BL2 conditions.[34,35]

Viruses were grown in 10-day-old embryonated chicken eggs. Stock strains generated by reverse genetics were diluted to $10^{5.5}$ egg infectious dose$_{50}$ and injected onto the allantoic membrane.

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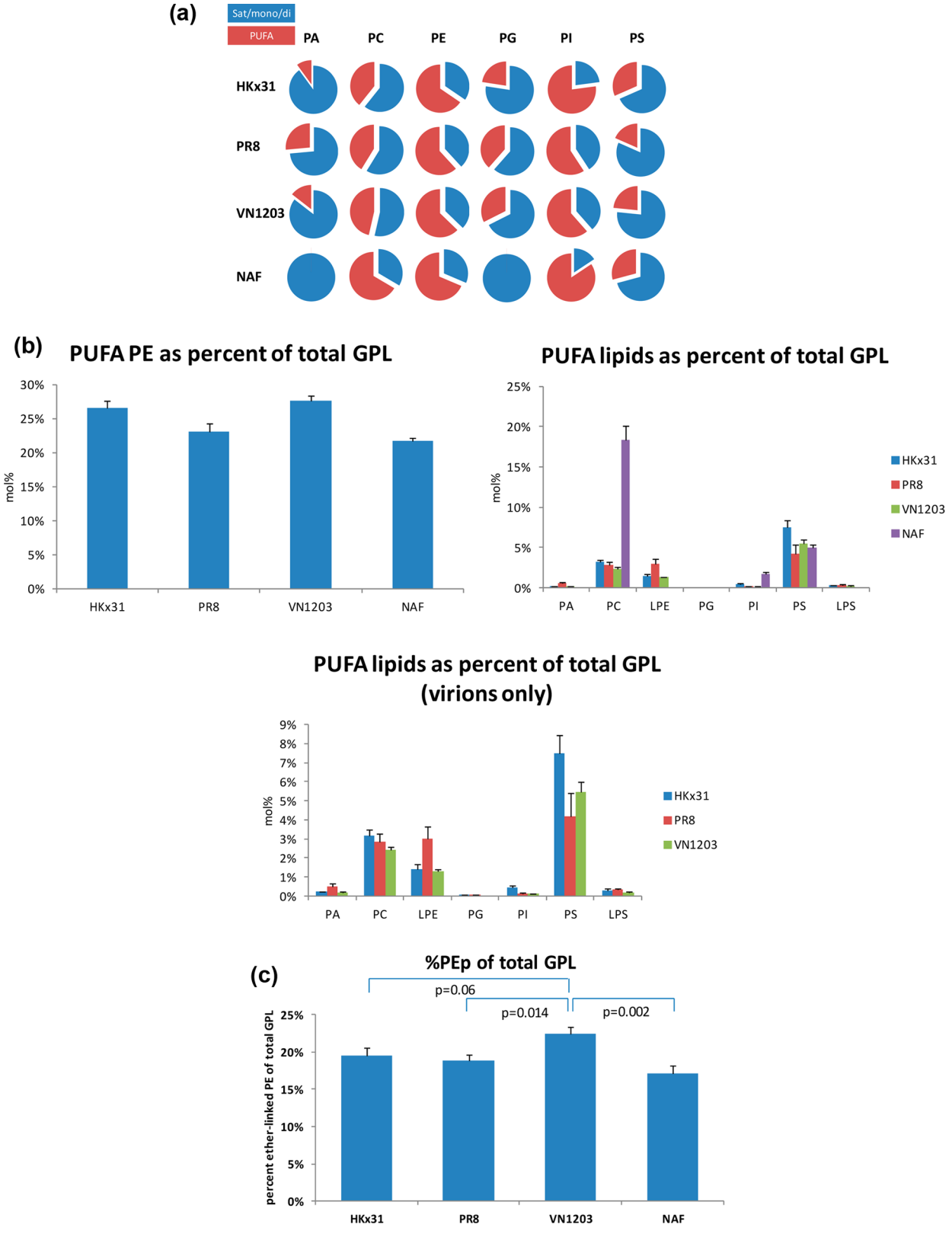

Figure 3. (a) Representation of proportion of polyunsaturated (red) versus saturated, monounsaturated, and diunsaturated lipids (blue) across virions (vertical) and glycerophospholipid classes (horizontal). (b) PUFA content by class in virions and NAF. (c) Plasmalogen ethanolamines as a fraction of total GPL for all viral strains and host.

Each individual preparation of virus was grown and purified separately.

After 48 h of incubation, eggs were chilled for 24 h at 4 °C and allantoic fluid collected. The subsequent fluid was cleared by centrifugation at 500*g*. Next, the material was concentrated by pumping through a Pellicon 2 ultrafiltration cassette (P2B100v01, Millipore). The concentrate was run across a discrete 25−70% sucrose gradient by ultracentrifugation at

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Table 2. Saturated FA-Containing Glycerophospholipid Distribution Comparison between Influenza Strains and Host (NAF) (Mole Percent)

	PA	LPA	PS	LPS	PG	LPG	PI	LPI	PC	LPC	PE	LPE
NAF									7		5.8	
VN1203	4.6	39.5	1.9	44.2	0	63.6		44.4	7.94	96.4	8.4	72.4
PR8	2.8	64	14.1	42	3.1	50		50	8.6	93	7.7	66.5
HKx31	4	100	0.9	37.4	1.7	60		55.6	11	91.7	5.1	64.6

27000 rpm for 1 h. The virus collects in a band at the interface and is removed by pipetting. A second continuous 25−70% sucrose gradient was run at the same speed. The harvested virus band was diluted in a sterile Tris−EDTA buffer (STE) and pelleted in the same buffer at 10000 rpm for 1 h.

Characterization of Virion Purity. Ten percent of the viral preparation was removed at the final spin stage after resuspension in STE. Plaque assays were performed on the resuspended virus. Viruses were added for 1 h to confluent monolayers of Madin−Darby canine kidney (MDCK) cells in 10-fold dilutions (10^{-5}−10^{-10}). The fluid was then discarded and replaced with agar containing 1 μg/mL L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). After incubation for 72 h at 37 °C, the agar overlays were removed, the cells were stained with crystal violet, and plaques were counted for virus titer calculation as plaque-forming units (PFU) per milligram of protein. Protein concentrations were tested by BCA assay (Pierce) according to the manufacturer's instructions. To generate a measure of virion purity, a ratio of PFU:protein was calculated and used for normalization between samples. Purity was also assessed by electron microscopy of the resuspended virus.[36]

Glycerophospholipid Extraction. Glycerophospholipids from purified virion pellets of different influenza strains were extracted using a modified Bligh and Dyer procedure.[37] Briefly, each pellet was homogenized in 800 μL of ice-cold 0.1 N HCl/CH_3OH (1:1) by vortexing for 1 min at 4 °C. Suspension was then vortexed with 400 μL of cold $CHCl_3$ for 1 min at 4 °C, and the extraction proceeded with centrifugation (5 min, 4 °C, 18000*g*) to separate the two phases. The lower organic layer was collected and solvent evaporated.

Extraction of phospholipids from NAF proceeded with the addition of 1 mL of ice-cold 0.1 N methanolic HCl and 1 mL of ice-cold $CHCl_3$ to 1 mL of NAF. Following 1 min of vortexing at 4 °C, layers were separated by centrifugation (5 min, 4 °C, 18000*g*) and the solvents from the lower layer evaporated. All procedures were performed quickly and at low temperature to prevent plasmalogen phospholipid degradation. The resulting lipid film was dissolved in 100 μL of isopropanol/hexane/100 mM NH_4COOH (aq) 58:40:2 (mobile phase A).

Mass Spectrometric Analysis. Quantification of GPLs was achieved by the use of an LC-MS technique employing synthetic odd-carbon diacyl and lysophospholipid standards and was based on standard curves, constructed from even-carbon species with spanning acyl chain length and degree of unsaturation. Typically, 200 ng of each odd-carbon standard was added per sample. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple-quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high-pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-μm particle size) using a gradient elution as previously described.[17,38] The identification of the individual species, achieved by LC-MS/MS, was based on their chromatographic and mass spectral characteristics compared to those of synthetic standards. This analysis allows identification of the two fatty acid moieties but does not determine the relative position on the glycerol backbone (*sn*-1 versus *sn*-2). All analyses were performed from multiple preparations of purified virions and NAF (n = 8), and mean data are reported in the figures and tables.

As a result of a comprehensive phospholipid profiling of purified virions from three different influenza virus strains with a range of pathogenicity in humans and NAF, we have identified differences in membrane lipid compositions. Biophysical properties of the virion are highly affected by its lipid composition and are reacting to the environmental conditions.[39] The literature indicates that virions with lower interferon expression (H5N1 is lower than H1N1 or H3N2) have higher replication rates and vice versa.[40,41] Host cells may be less able to defend against virions that evoke interferon responses. Related early lipid changes may be critical to membrane dynamics through factors such as changes in plasmalogen ethanolamines, generation of lysolipids, and increased SM synthesis. Thus, membrane organization and architecture are highly dependent on the lipid environment during viral infection and suggest that viral replication can be influenced by modifying its structure. For instance, inhibitors of lipid biosynthesis (such as fatty acid synthase, ethanolamine kinase, and sphingosine kinase) can be perceived as pharmacological targets for developing effective antivirals.

In addition, GPLs themselves can serve as potential therapeutics. Volker and colleagues[42] showed that two strains of influenza A bind to palmitoyl-oleoyl-phosphatidylglycerol, which is a minor component of pulmonary surfactant. These findings suggest that the addition of this molecular species as part of a surfactant supplement might be an effective treatment approach against viral infections.[43] Similarly, it was recently demonstrated that changes in host cell lipid composition play key roles in influenza infectivity. Our group recently demonstrated that phospholipase D (PLD) is activated in host cells following presentation with influenza virus, and the PLD2 isoenzyme plays a major role in assisting the virus escape from immune defenses.[44] Allosteric modulators of PLD greatly decelerate the kinetics of entry, and data suggest that PA may be a novel target for the development of new antiviral therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00040.

Tables with identified phospholipid species in three strains of influenza and NAF, table with quantitated lipid species in the influenza strains and NAF, figure comparing long-chain fatty acid compositions (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ESI-MS, electrospray ionization mass spectrometry; FA, fatty acid; GPL, glycerophospholipids; LC-MS, liquid chromatography−mass spectrometry; MS/MS, tandem mass spectrometry; NAF, noninfected allantoic fluid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEp, plasmalogen ethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin

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