Chapter 14

Metabolomics and Lipidomics

Yet More Ways Your Health Is Influenced by Fat

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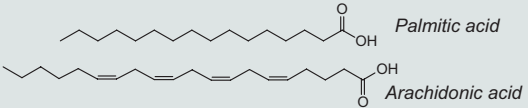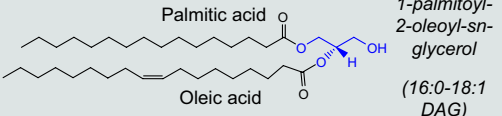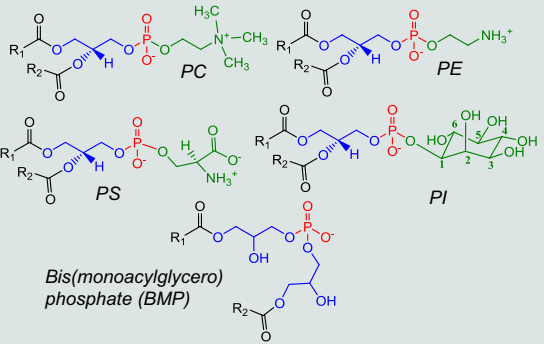
1. INTRODUCTION

Cellular metabolism is comprised of the chemical reactions that occur in living cells. Broadly, these reactions can be divided into catabolic reactions that convert nutrients to energy and anabolic reactions that lead to the synthesis of larger biomolecules. The reactants and products of these chemical reactions are metabolites. The flow of genetic information from DNA (genome) to RNA (transcriptome) to protein (proteome) described by the Central Dogma leads to production of the metabolome. The composition of the metabolome is dynamic and reflects expression of the genome under specific conditions. Metabolomic changes induced by viral infection are the integration of virus-induced changes in both host gene expression and host protein function.

As early as the 1950s, Seymour S. Cohen advanced the idea of viral replication as a series of biochemical reactions whose reactants and products are amenable to dissection, writing: "Many of the most important biological questions have been rephrased as chemical problems. Questions now

are being posed concerning the nature of the building blocks and the pathways of their biosynthesis. The time course of infection, duplication, and virus liberation is being dissected minute by minute in terms of the molecular transformations occurring in these systems." Cohen advocated that the tools of chemistry would enable understanding of viral processes at the molecular level. In the 60-odd years since Cohen first referred to this area of investigation as "chemical virology," advances in analytical methods have facilitated increasingly precise knowledge of the interaction of viruses with host metabolism.

The major classes of metabolites include amino acids, carbohydrates, nucleotides, lipids, coenzymes, and cofactors. These classes of compounds encompass an enormous diversity of molecular structures, physico-chemical properties, functions, and abundances. Due to the analytical challenges posed by this diversity, most studies require division of the metabolome into subsets of metabolites. The most common distinction is that between hydrophilic (polar) metabolites and hydrophobic

TABLE 1 Classes and Functions of Lipids

Lipid Class	Examples	Function
Fatty Acids/Fatty Acyls		
Carboxylic acids and their derivatives	*Palmitic acid*	Energy storage
Hydrophobic tail varies in length and degrees of unsaturation	*Arachidonic acid*	Building block for other lipids
Glycerolipids		
Glycerol core (blue) with one, two, or three fatty acyl groups to form mono-, di-, and tri-acylglycerides (MAG, DAG, TAG)	Palmitic acid / Oleic acid / *1-palmitoyl-2-oleoyl-sn-glycerol* / *(16:0-18:1 DAG)*	Energy storage
		DAG are also second messengers in signal transduction and pre-cursors for prostaglandin synthesis
		TAG are used for energy transport
Glycerophospholipids		
DAG backbone (blue) with phosphate at third alcohol (red)	PC, PE, PS, PI structures	PC, PE, PS are structural components of membranes
Phosphate (red) esterified with choline, ethanolamine, serine, or inositol (green) results in phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), and phosphatidylinositols (PI), respectively		PE and PS induce membrane curvature, as do MAGs like BMP
Acyl groups are represented as "R_1" and "R_2" and vary in length and degree of unsaturation	*Bis(monoacylglycero) phosphate (BMP)*	PS and PI serve as docking sites in membranes for signaling proteins
		PI are precursors of secondary messengers and form a "lipid code" signifying membrane identity
Sphingolipids		
Sphingosine base is the long-chain aliphatic amine backbone (R_1)	Sphingosine	S1P are extracellular signaling molecules
Ceramides (CER) are the sphingosine base acylated at the amine (R_2)	Sphingosine-1-phosphate (S1P) / Ceramide (CER) / Sphingomyelin (SM)	CER and SM are structural components of membranes that can induce formation of lipid-ordered domains ("rafts") and membrane curvature
Sphingomyelin (SM) corresponds to a ceramide conjugated to phosphatidyl-choline (green) at the second alcohol		
R_2 varies in length and saturation		

Sterols

Sterols are defined by a four-ringed structure		Cholesterol is the structural component of membranes
Rings are labeled A, B, C, D		Packing of cholesterol with SM induces lipid-ordered domains important for signal transduction and viral assembly
Sterol esters are formed by esterification with fatty acids		Sterols are also signaling molecules
		Sterol esters are used for energy storage

Cholesterol

Desmosterol   25-hydroxycholesterol

Bioactive Lipids

Bioactive lipids are derived from arachidonic acid and related molecules		Signal transduction via G-protein-coupled and peroxisome proliferator-activated receptors
These include prostaglandins, thromboxanes, lipoxins, leukotrienes, resolvins, protectins, and maresins		Pro- or anti-inflammatory effects

Arachidonic acid

Prostaglandin E$_2$ Lipoxin A$_4$

(nonpolar) metabolites. Polar metabolites are soluble in aqueous solutions and include most sugars, purines and pyrimidines, nucleotides and nucleosides, acyl carnitines, organic acids, hydrophilic acids, amino acids, and phosphorylated compounds. These metabolites include most of the reactants and products involved in cellular respiration (e.g., glycolysis, the TCA cycle, or the pentose phosphate pathway) and in the production of building blocks for synthesis of large biopolymers such as DNA, RNA, proteins, and oligosaccharides. The nonpolar or hydrophobic metabolites are traditionally referred to as lipids. These metabolites function in energy storage, membrane structure, and signal transduction. Reflecting the diversity and complexity of lipid structures and functions (Table 1), the study of lipid metabolites has developed as a subfield within metabolomics. Thus, the complete metabolome formally includes both hydrophilic and hydrophobic metabolites, but the term "metabolome" is now frequently used to refer to the hydrophilic metabolome and the term "lipidome" is used to refer to hydrophobic metabolites. This chapter provides background and a framework for examining the role of lipid metabolites in viral processes and highlights general themes in our current understanding of the function of lipids in viral replication and pathogenesis.

Viruses require hydrophilic and lipid metabolites from the host. First, viruses are unable to manufacture the primary metabolites required for synthesis of new virions. The nucleotides, amino acids, and lipids that are structural components of the viral particle are all commandeered from the host cell. Likewise, cellular respiration provides the fuel required to drive the energetically costly process of viral replication. The effects of many viruses on metabolic pathways that produce the building blocks and energy needed for viral replication are well-documented. Although these perturbations are thought to support viral replication, the underlying molecular mechanisms are still the subject of much investigation. Second, many viruses use specialized membranes for viral entry, gene expression, genome replication, and assembly. The distinct morphology and functional properties of these membranes presumably reflects their specialized composition, which has been optimized for the viral process that they support. Analysis of the chemical composition and biophysical properties of these membranes, as well as the molecular mechanisms underlying their biogenesis, is therefore central to understanding their function in viral processes. Third, signal transduction by bioactive lipid metabolites plays a major role in the host response to viral infection and, in many cases, in pathogenesis. Identification of bioactive lipids associated with viral replication or the host inflammatory response holds promise for the identification of biomarkers of infection and disease progression as well as for identifying potential points for pharmacological intervention (Box 1).

Metabolomic profiling enables the systematic identification of perturbations of metabolism that arise from the interaction of viral pathogens with the host. Differences in metabolomic profiles reflect changes in protein function. This contrasts and complements the changes in gene expression and protein abundance detected by transcriptomic and

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Box 1 Metabolomics for the discovery of antiviral targets

Metabolic enzymes and other metabolite-binding proteins are an attractive source of potential antiviral targets. First, metabolic reactions are essential to the infectious cycle of all viruses. Synthesis of new virions is itself comprised by the chemical conversion of nucleotides, amino acids, and lipids into progeny virions. Host-catalyzed chemical reactions that lead to the synthesis of these basic building blocks, and to the release of energy, have direct impacts on viral replication. Second, since the natural function of metabolite-binding sites on proteins is to bind to small molecules, these sites are generally reasonable molecular targets for drug discovery efforts. Indeed, many existing drugs mimic the interaction of naturally occurring metabolites with their respective enzymes and receptors. These "anti-metabolites" act by preventing utilization of the natural metabolite. Traditional antivirals targeting viral polymerase and protease activities are good examples of drugs that act by this mode. They inhibit the natural catalytic function of these enzymes by binding to the active site or to allosteric sites. These antiviral drugs are selective because they target metabolic reactions unique to the virus. Although this approach has produced some of the most successful drugs on the market today, it is inherently limited because viral genomes encode a very limited number of metabolite-binding proteins, and because resistance can develop through mutations that affect drug binding without affecting catalytic function. In addition, the selectivity of these drugs for their specific viral targets usually limits their use to a narrow spectrum of closely related viruses.

Targeting essential metabolic reactions catalyzed by host factors offers an alternative antiviral strategy. Viruses are fully dependent upon host metabolism and do not themselves encode the enzymes needed to produce the metabolites required for their own replication. Inhibition of a metabolic reaction critical for viral replication is therefore unlikely to be overcome by direct mutations of the viral genome. Likewise, the centrality of host metabolism to the viral infectious cycle makes it likely that phylogenetically related viruses have shared dependencies on specific metabolic pathways. Although the host proteome is replete with enzymes and proteins that bind to small-molecule metabolites, the challenge is to identify those that can mediate antiviral activity without affecting normal host cell function. As advances in analytical methods have enabled both broader and deeper surveys of virus-induced perturbations of host metabolism, identification of targets that mediate adequately selective antiviral effects has come closer to reality.

proteomic studies. The combination of these approaches provides the fullest picture of virus–host interactions occurring at both the molecular and pathway levels.

2. DEFINING THE ROLE OF THE LIPIDOME IN VIRUS–HOST INTERACTIONS

The goal of lipidomic analysis is the quantitative characterization of all lipids in a biological system, including the dynamic changes that occur in response to stimuli and the physiological consequences of these changes. This is technically challenging due to the structural diversity of lipid molecules and the inability to predict or infer lipid structure based on genomic, transcriptomic, or proteomic data. Lipidomic analysis is also challenging because the abundance of individual species can vary over a wide range. Phospholipids are so abundant that they constitute up to 50% of the total mass of lipids in the cell, whereas prostaglandins and other bioactive signaling lipids may be present at only picomolar concentrations. No single analytical method is sufficient to capture the full lipidome of a biological system, and as with other "omics" approaches, the methods used for quantitative analysis of large lipidomic data sets affect interpretation of the data. Strategies currently being used to examine the interaction of viruses with host lipids generally fall into one of three categories. First, there are classical reductionist approaches using metabolic labeling and biochemical reconstitution experiments to examine the potential function of a limited set of specific lipids in viral processes. Second, it is possible to infer roles for different classes of lipids through transcriptomic and proteomic studies that detect changes in the expression of host enzymes responsible for the synthesis, metabolism, and trafficking of these lipid classes. Third, global analytical chemistry methods allow direct detection and quantification of specific lipid species that change as a function of viral infection. This section highlights the types of biological questions that can be answered using these different approaches.

2.1 Reductionist Approaches

One of the first demonstrations that viruses affect host metabolism arose from the work in the 1950s of Hilton Levy and colleagues, who monitored the appearance of lactic acid in tissue culture supernatants and demonstrated that glycolysis is increased very early in poliovirus infection (Baron and Levy, 1956). Additional studies documented virus-induced changes in the uptake of isotopically labeled amino acids, phosphate, and other nutrients, suggesting perturbation of their respective metabolic pathways. These types of experiments were restricted by existing knowledge of specific metabolic reactions of interest. Efforts to "profile" virus-induced metabolomic changes in an unbiased manner were limited by the analytical methods available for detection and quantification of a finite set of metabolites (or metabolite classes).

Analogous reductionist approaches that focus on a particular metabolic pathway are useful today in corroborating discoveries made using newer, more discovery-based global methods (discussed below) and in examining the biochemical functions of individual lipid species in viral replication. Loss-of-function experiments that detect a viral phenotype when a specific lipid class is depleted from a host cell can be used to establish the essentiality of the depleted lipid for a viral process. Elegant studies of brome mosaic virus (BMV) replication in yeast led to the discovery that BMV RNA replication is blocked in yeast strains that have a reduced ratio of unsaturated to saturated fatty acids (UFAs and SFAs, respectively) due to mutations in the gene encoding $\Delta 9$ fatty acid (FA) desaturase, even when these mutations have no effect on cell growth (Lee et al., 2001). The antiviral effect of UFA depletion on BMV RNA replication is correlated with depletion of UFAs from replication membranes and decreases in membrane fluidity (Lee and Ahlquist, 2003). Rescue of BMV RNA replication by supplementation of growth medium with UFAs suggests a functional role of UFAs in this process. As another example, studies of Flock house virus (FHV) replication in a cell-free system allowed systematic examination of various glycerosphingolipids, sphingomyelin, and cholesterol in FHV RNA replication and the discovery that positive-strand RNA synthesis is promoted by glycerophospholipids. This effect varies depending on the length of the acyl chain and its degree of saturation (Wu et al., 1992). The major limitation of the approaches used to make these discoveries is that most viral processes cannot be recapitulated in genetically tractable organisms or in cell-free systems. A notable exception to this is the fusion step of viral entry, which has been examined for many enveloped viruses through the use of synthetic liposomes of defined composition and size.

2.2 Inferring Metabolomic Changes from Genomic or Proteomic Profiling

Examination of changes in host gene expression has become a useful tool for discovering how viruses affect metabolism. These studies implicitly assume that changes in enzyme abundance correspond to changes in the quantity or quality of metabolic output and that these changes are functionally relevant for viral replication, pathogenesis, or the host response. Systematic identification of virus-induced changes in steady-state host gene expression can now be routinely performed using global transcriptomic and proteomic methods (see Chapter 11). Conversely, RNA interference (RNAi) experiments that look for loss of replication upon depletion of a given host factor are also widely used to validate candidate pathways identified through transcriptomic and proteomic profiling studies and for the de novo discovery of metabolic pathways that are important for viral replication. Profiling and RNAi screening approaches have

also been used to study viral pathogenesis with the goal of identifying pathways, and even specific host factors, that are critical for viral pathogenesis and that are candidate targets for antiviral intervention. For example, recent comparative transcriptomic analyses discovered that increased transcription of host lipid metabolic genes regulated by the liver X and retinoid X receptors (LXR/RXR) is characteristic of low pathogenicity influenza virus infections, whereas these changes are not observed during infection with H7N9 or other pathogenic avian influenza viruses or the highly pathogenic 1918H1N1 human influenza virus (Josset et al., 2012; Morrison et al., 2014).

Although these types of studies have been powerful tools in identifying virus-induced perturbations of the host cell, they provide an incomplete picture of a virus' effect on the host metabolome. Expression of many metabolic enzymes is regulated posttranscriptionally; moreover, the function of many enzymes is regulated posttranslationally through modifications that affect enzyme activity, oligomerization, or localization. Consequently, transcriptomic profiling experiments cannot predict all changes in protein abundance, and proteomic profiling experiments cannot detect changes in the activities and output of host metabolic enzymes. For related reasons, it is difficult to predict how RNAi-mediated depletion of a given metabolic enzyme affects the flux and molecular output of the pathways in which it functions. The potential limitations of relying upon only one type of profiling approach are illustrated by transcriptomic, proteomic, and lipidomic analyses of hepatitis C virus (HCV) infection. RNA profiling of HCV infection in vivo identified increased abundance of transcripts involved in glycosphingolipid synthesis and signaling by the serum response element binding protein (SREBP) pathway that are correlated with the onset of viremia (Su et al., 2002), suggesting that these pathways are important for HCV replication. Changes in the abundance of transcripts involved in lipid metabolism genes later in infection are correlated with steatosis and oxidative stress and thus are proposed to reflect a function of these gene products in pathogenesis (Smith et al., 2003; Bigger et al., 2004). A separate proteomic profiling of HCV infection in cell culture (Diamond et al., 2010) identified significant changes in enzyme abundance that were not predicted by the earlier transcriptomic studies. For example, changes in the abundance of proteins involved in energy production (i.e., glycolysis, the pentose phosphate pathway, and the citric acid cycle) early in infection were observed, suggesting that HCV increases energy production to meet the demands of viral replication. Parallel changes in the abundance of enzymes responsible for fatty acid oxidation have been interpreted to reflect a requirement for energy or increased acetyl-CoA for membrane synthesis. The perturbations detected by proteomic analysis likewise represent only a portion of the metabolomic changes induced by HCV since a parallel lipidomic profiling study detected HCV-induced enrichment of certain phospholipids and decreases in other

phospholipid, sphingomyelin, and triacylglycerol species early in infection. Since not all lipids of these respective classes are uniformly affected, lipidomic analysis in this case enabled a higher resolution understanding of HCV's effects on lipid metabolism. This in turn provides an opportunity to identify particular molecules within a given pathway or lipid class that have a specific and optimized function in viral replication, pathogenesis, or the host response.

2.3 Global Analytical Methods

2.3.1 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy monitors the absorption and emission of electromagnetic radiation by atomic nuclei. These occur at specific "resonance" frequencies affected by the local environment and thus provide data regarding structure and dynamics. Since NMR experiments do not require destruction or modification of the sample, the method is amenable to high-throughput analysis of biological samples and monitoring metabolic flux. Studies monitoring 50 or fewer species present at concentrations in the micromolar to millimolar range are the practical limit for metabolomic experiments due to resonance overlap and the limits of magnet strength. Although NMR-based metabolomic methods have not been widely used to study lipid function in the context of viral infection, they hold promise as a "fingerprinting" technique to discover biomarkers that are indicative of viral infection or disease state.

2.3.2 Mass Spectrometry

Mass spectrometry (MS) measures the molecular weight of a molecule by ionizing it and then measuring how its trajectory through a vacuum is affected by electric and magnetic fields. A mass spectrum plots the signal intensity (abundance) of a given ion as a function of its mass-to-charge (m/z) ratio, which enables measurement of molecular weight and also can provide structural information. Liquid or gas chromatography (LC or GC) is commonly used to separate molecules so that those with the same m/z ratio enter the spectrometer at different times. Electrospray ionization is the most commonly used method for lipidomic studies because it does not have a major structural bias and because it generates intact molecular ions and therefore enables ion abundance to be taken as a measure of the abundance of the parent metabolite. The combination of unique retention times and m/z ratios measured by quadrupole or Orbitrap mass spectrometers permits the routine quantitation of thousands of peaks in a single metabolomics profiling experiment with sensitivities as low as the femtomolar range.

MS-based profiling experiments can be performed in "targeted" or "untargeted" modes. Targeted studies focus

on a limited set of metabolites for which retention times and m/z ratios have already been established using authentic standards. Untargeted profiling experiments monitor all detectable ions and use computational tools (e.g., XCMS or MetAlign) to identify differences in ion abundance between different samples. Assigning a structure to an ion of interest then requires tandem LC-MS/MS or other analytical experiments to compare the ion of interest with authentic standards. Targeted profiling provides a rapid and rigorous way to confirm the general effects of viral infection on a class of lipids or a lipid pathway, to test hypotheses generated from gene expression and proteomic analyses or untargeted metabolomic profiling, and to obtain higher resolution data regarding specific changes within pathways of interest. Untargeted analysis is more suited to the discovery of specific lipids that may be important in viral infection. As discussed above, targeted lipidomic profiling of HCV has been useful in interpreting and validating HCV-induced changes in lipid homeostasis inferred from transcriptomic and proteomic profiling studies. An independent untargeted study corroborated the targeted profile but in addition identified 26 distinct lipid species whose abundance changed by threefold or greater during HCV infection (Rodgers et al., 2012). One of these changes was a greater than 10-fold increase in desmosterol, a penultimate intermediate in cholesterol synthesis that is normally not abundant in the host cell. The discovery that HCV replication is inhibited under conditions in which desmosterol synthesis is blocked and rescued upon the addition of exogenous desmosterol (versus other sterols) suggests that this molecule has a specific function in HCV replication. *A priori*, there was little rationale for including desmosterol in the targeted study since this biosynthetic pathway is highly regulated and driven toward its end product, cholesterol. In another example, untargeted metabolomic profiling led to the discovery that *N*-acetyl-aspartate is the most up-regulated metabolite in cells infected with human cytomegalovirus (HCMV) (Rabinowitz et al., 2011). This change was unlikely to be detected in targeted studies because the synthetic route for this metabolite is unclear and it does not appear on standard metabolic maps or on lists of targeted metabolites.

An important distinction in MS-based studies is that between steady-state and flux analyses. Steady-state measurements quantify the abundance of metabolites at a given point in time and compare data collected from two or more different biological conditions (e.g., mock vs virus infected, high- vs low-pathogenicity strains, or early vs late infection). Any changes detected may reflect differences in synthesis or turnover. As illustrated by the aforementioned untargeted profiling studies of HCMV and HCV, this approach can be especially useful in detecting metabolites that are relatively rare in uninfected cells and that undergo large changes in abundance in the presence of a virus. Smaller fold changes in highly abundant molecules may also correspond to functionally significant changes in the total lipid composition of the cell.

Viruses may also affect reaction rates in a given biochemical pathway without altering steady-state metabolite abundance. For example, increased rates of cellular respiration during viral infection may not affect the steady-state abundance of ATP because it is consumed to fuel the energy demands of viral replication. Metabolic flux analysis directly measures changes in reaction rates for a given biochemical pathway. Pioneering work by Rabinowitz and colleagues has demonstrated the utility of LC-MS and isotopically labeled nutrients to profile kinetic flux through metabolic pathways (Rabinowitz et al., 2011). This allows discovery of specific pathways affected by viral infection and can also provide insights into underlying mechanisms. For example, comparative flux analysis of ^{13}C-labeled glucose in cells infected with HCMV or herpes simplex virus 1 (HSV-1) has revealed that both viruses induce the TCA cycle; however, HCMV-infected cells produce citrate with two ^{13}C atoms (reflecting synthesis via glycolytic flux) whereas HSV-1-infected cells produce citrate with three ^{13}C atoms (reflecting synthesis via anaplerotic flux) (Vastag et al., 2011) (Figure 1). The effect of each virus is distinct and their differences would not be detectable by steady-state measurements of transcript, enzyme, or even metabolite abundance. Analogous "fluxomic" analysis of lipid metabolic pathways may provide insights into the biogenesis of membranes utilized by viruses during replication.

3. LIPIDS IN VIRAL REPLICATION AND PATHOGENESIS

The membranes used in viral replication presumably have a specific composition that has been evolutionarily optimized to support the associated viral process; however, we know little about the molecular structures of specific lipids that function in viral replication and pathogenesis. Here, we discuss some of what is known about the function of lipids in different viral processes. Specific examples, primarily from studies of RNA viruses, are provided as examples but do not represent a comprehensive review of the current literature.

3.1 Lipids in Viral Entry

Lipids are central to the process of viral entry. First, glycosphingolipids and other lipids on the plasma membrane serve as entry factors mediating the initial attachment of many viruses to the host cell. Second, viral entry requires that the viral genome transit from the exterior of the host cell and past the plasma membrane to an interior cellular compartment. This requires fusion of the viral membrane with a cellular membrane (enveloped viruses) or penetration of a cellular membrane (nonenveloped viruses). Relatively more is known about fusion; however, both membrane fusion and
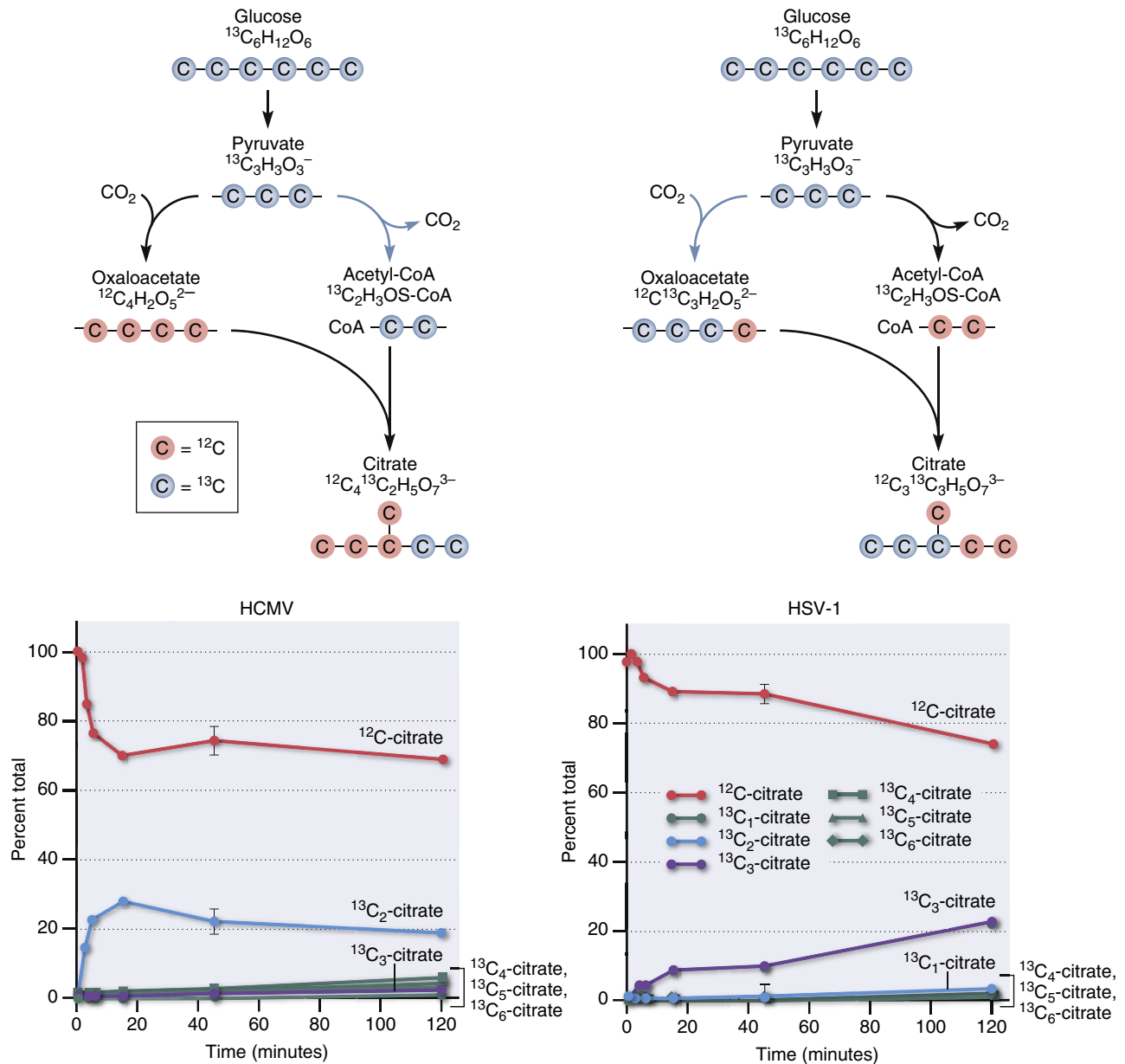
FIGURE 1 Flux analysis provides information on reaction rates and metabolic pathways affected by viruses. Herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV) infections both cause increases in the abundance of intermediates from glycolysis and the TCA cycle. Quantification of changes in metabolite abundance does not provide information on how these effects are produced (i.e., due to changes in influx, efflux, or both). By flux analysis monitoring conversion of ^{13}C-labeled glucose to citrate, Rabinowitz and colleagues determined that HSV-1 causes an increase in anaplerotic flux to the TCA cycle via pyruvate carboxylase (increased citrate with three ^{13}C-labeled carbons). In contrast, HCMV increases glycolytic flux, as evidenced by the accumulation of citrate labeled with two ^{13}C. *From Rabinowitz et al. (2011).*

penetration are affected by the lipid composition of target membranes.

3.1.1 Membrane Fusion

Fusion requires that the viral lipid bilayer transition from strongly positive to strongly negative curvature. Association of the viral envelope protein with both the viral and target membranes results in coupling of its structural rearrangements to membrane distortions that lower the energy barrier for reorganization of the two membranes as an initial hemifusion intermediate, and subsequently a fully fused product with a fusion pore that permits release of the viral genome (Figure 2). Physiological triggers, such as binding to a receptor or co-receptor at the plasma membrane (e.g., HIV) or exposure to acidic pH in the endosome (e.g., influenza virus), ensure that structural rearrangement of the envelope protein does not initiate prematurely.
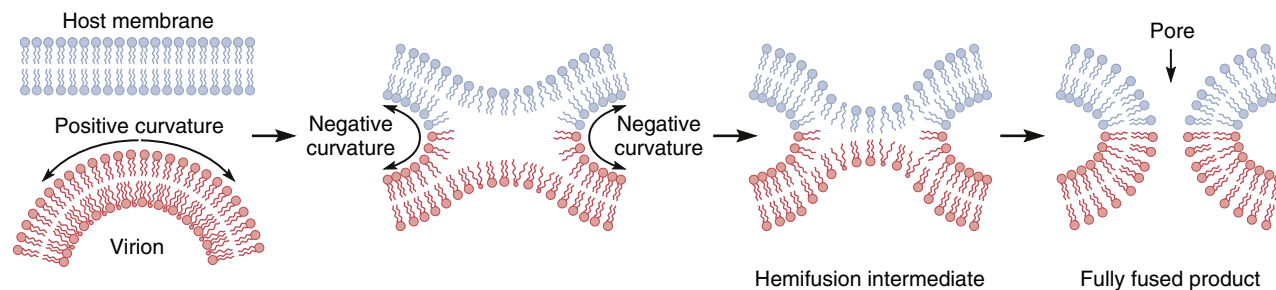
FIGURE 2 Membrane curvature during membrane fusion in viral entry. Fusion of viral and cellular membranes during enveloped virus entry requires the induction of membrane curvature to form, first, a hemifusion intermediate in which lipid mixing between outer leaflets has occurred and subsequently, a fully fused product. Lipid head group size and charge as well as interactions with proteins contribute to the induction of the necessary curvature (Text Box 2).

3.1.2 Membrane Composition

The functional properties of membranes, including membrane curvature, fluidity, elastic free energy, and lipid packing are determined by their lipid composition (Box 2). A long-standing hypothesis has been that the compositions of the viral lipid bilayer and the host target membrane are key determinants of the efficiency of fusion and other steps in viral entry.

Studies of specific viruses in biochemical reconstitution and cell-based models have revealed richness in the effects of lipid structure and function on membrane fusion during viral entry. Many viruses, including alphaviruses, HIV, HSV-1, HCMV, and influenza virus, require cholesterol (or other sterols) for efficient fusion (Box 3); however, there are differences in how and where cholesterol functions during entry (viral membrane, cellular membrane, or both). HIV entry requires cholesterol- and sphingolipid-rich lipid rafts in both the viral membrane, where they stabilize virion structure and infectivity, and in the plasma membrane, where they facilitate clustering of the CD4 receptor and CCR5/CXCR4 co-receptor. In contrast, Semliki Forest virus requires cholesterol because the conformational changes in the viral E1 protein that catalyze fusion cannot occur without its interaction with cholesterol (Umashankar et al., 2008). Influenza A virus entry requires cholesterol in the viral membrane but not in the target membrane, although the functional basis for this requirement is not well-understood.

Beyond requirements for cholesterol and sphingolipids, the fusion of many viruses may be regulated by the presence of compartment-specific lipids. Live-cell imaging studies have demonstrated that dengue virus enters via clathrin-mediated endocytosis and fuses in Rab7-positive late endosomes (van der Schaar et al., 2008), yet conformational changes in the dengue E protein can be triggered in vitro at the moderately acidic pH encountered by the virus in early endosomes. An additional requirement for negatively charged lipids in the late-endosomal membrane may explain the apparent delay between the initiation of fusion and release of the nucleocapsid. This is supported by the observation that negatively charged lipids, such as

phosphatidylserine or bis(monoacylglycero)phosphate, increase the fusion of dengue virus with synthetic liposomes (Zaitseva et al., 2010; Nour et al., 2013). Negatively charged lipids may be required to trigger conversion of a "restricted hemifusion" intermediate to the fully fused product. Alternatively, the virus may initially fuse with small endosomal carrier vesicles in the early endosome and back-fusion with the late endosome requires negatively charged lipids.

3.2 Lipids in Viral Genome Replication

For many viruses, genome replication occurs on specialized membranes; however, the specific lipids in these membranes are generally not well characterized. Recent progress in studying the specialized replication compartments used by positive-sense RNA viruses highlights a growing appreciation for the function of membranes in this viral process.
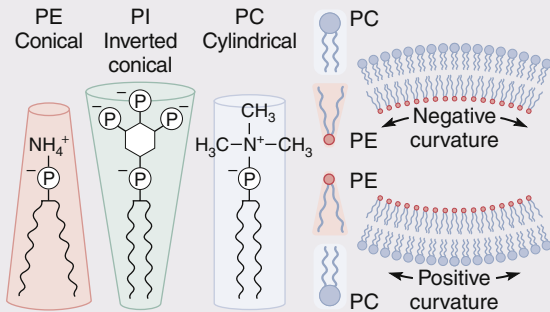
3.2.1 Genome Replication of RNA Viruses

Localization of viral RNA replication to specialized replication compartments—essentially, virus-specific organelles—serves several functions. First, membrane localization promotes RNA replication by concentrating the reactants, catalysts, and cofactors required for RNA replication. Second, interaction of the macromolecular replication complex with the interior membrane of the compartment may scaffold factors in specific conformations and orientations necessary for activity. Third, sequestration of the viral genome within the compartment provides a mechanism for regulating the processes of replication, transcription, translation, and assembly that all compete for use of the genomic RNA. Spatial restriction of genome replication to the compartment also serves to shield the process from detection by the host response.
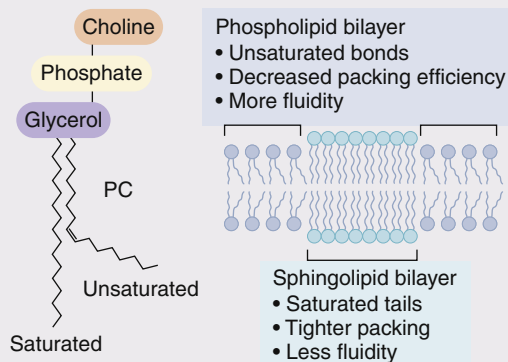
RNA viruses vary greatly in their use of different subcellular host membranes, including those of the rough endoplasmic reticulum (flaviviruses, picornaviruses, SARS-coronaviruses), mitochondria (nodaviruses), and plasma
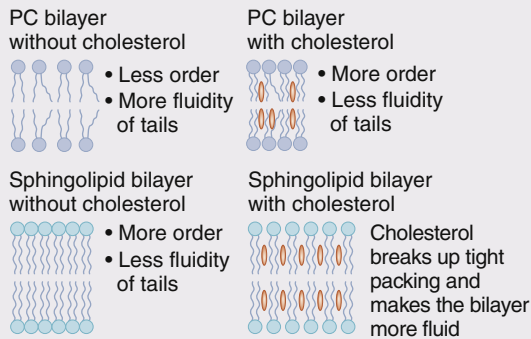
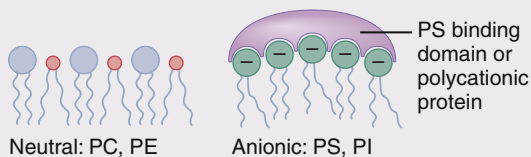## Box 2 Lipid structure and function in membranes

(A) Head group size

(B) Hydrophobic tail structure

(C) Interaction with sterols

(D) Membrane surface and head group charge

liquid-ordered domains (also known as "lipid rafts") that have decreased fluidity relative to the endoplasmic reticulum (ER), whose mobility is associated with the low abundance of order-promoting lipids. Although the chemical composition of most membranes that support viral processes has not been characterized, analysis of the specific lipid molecules in viral membranes is expected to provide insight into their specialized function in viral replication.

(A) Head group size The relative sizes of head groups and hydrophobic tails of glycerophospholipids affect the overall shape of the lipid as well as the thickness and curvature of the bilayer. Phosphatidylcholines (PC) have relatively large head groups compared to phosphatidylethanolamines (PE), and these differences directly affect the shape of individual molecules and the propensity of these lipids to assemble into lipid bilayers and to induce membrane curvature. PC spontaneously form lipid bilayers that exist in a liquid crystalline state at physiological temperature. The smaller, pH-titratable head group of PE gives these lipids a cone shape that imposes lateral stress on the bilayer and that can promote negative membrane curvature.

(B) Hydrophobic tail structure The structures of the hydrophobic tails of glycerophospholipids and sphingolipids also influence the width and fluidity of membranes. Molecular features, such as *cis*-double bonds, reduce packing density and enhance membrane fluidity. Lipids with fully saturated tail groups pack more densely, resulting in decreased membrane fluidity. An important example of this is the tight packing of sphingolipids, which generally have fully saturated acyl chains or *trans*-double bonds. The taller, narrower shape and tighter packing of ceramides and other sphingolipids relative to their phospholipid counterparts promotes the formation of ordered domains (lipid rafts) that may be used by viruses at the plasma membrane and other sites.

(C) Interaction with sterols Cholesterol and other sterols in the lipid bilayer can alter packing density and membrane fluidity by packing against acyl chains in the hydrophobic tail groups. This can be critical in maintaining the fluidity of sphingolipid bilayers, which can otherwise form a solid–gel phase. Conversely, ordered packing of acyl chains around the fused four-ring structure of cholesterol reduces the entropy of phospholipid bilayers, leading to decreased mobility of the bilayer as well as increased membrane thickness and reduced membrane permeability.

(D) Membrane surface and head group charge The surface charge of membranes is important for interactions with proteins. The anionic head groups of phosphatidylserines and phosphatidylinositols interact with a variety of proteins. The head group of PC is zwitterionic over a wide range of pH resulting in bilayers that have no net charge. The head group of PE is zwitterionic, but can become anionic upon deprotonation of the ammonium ion; its charge and interactions with proteins can therefore be regulated by pH.

The physicochemical properties of membranes are dictated by their lipid composition, which is optimized for specific biological functions. This is evidenced by the striking differences in composition and function of different subcellular membranes (Holthuis and Menon, 2014). The plasma membrane is rich in saturated fatty acids, sphingolipids, and cholesterol, all of which contribute to the formation of

Box 3 Cholesterol in viral infections

The importance of sterols in viral infection is evident in the sensitivity of many viruses to perturbation of sterol homeostasis and the repressive effect of the antiviral interferon response on the sterol biosynthetic pathway. In addition to its importance in membrane structure and function, cholesterol is important as a biosynthetic precursor of hormones, vitamins, and other lipid-signaling molecules. Signal transduction by downstream metabolites of sterols, such as the oxysterols and vitamin D, has been increasingly implicated in both the innate and adaptive immune responses to viral pathogens. Sterols that occur upstream of cholesterol were previously thought to exist only as biosynthetic intermediates, but they are now known to have additional functions in signal transduction. For example, desmosterol, a penultimate intermediate of cholesterol, is an activator of the liver X receptor (LXR) and the dominant ligand of LXR in macrophage foam cells, where it activates and inhibits transcription of LXR and SREBP target genes, respectively, and suppresses expression of inflammatory genes (Spann et al., 2012).

Enteroviruses cause dynamic changes in cholesterol trafficking and metabolism (Ilnytska et al., 2013). Early in infection, cholesterol internalization is increased while cholesterol stored as esters in lipid droplets is concomitantly decreased. Ectopic expression of the viral 2BC protein alone is sufficient to trigger this increase in free intracellular cholesterol, much of which appears to be trafficked to sites of viral RNA replication via Rab11-positive recycling endosomes. Inhibition of viral replication when cholesterol trafficking is blocked suggests that these dynamic changes in trafficking are functionally significant for the virus. For example, cholesterol depletion appears to affect proteolytic processing of the viral polyprotein. Although the exact mechanisms underlying the effects of enteroviruses on cholesterol trafficking still need to be elucidated, it is known that virus-induced perturbations of PI4P and cholesterol metabolism and trafficking (Figure 4) are not mediated by changes in host gene expression but rather by posttranslational mechanisms affecting host protein function and localization.

Studies of cholesterol function in viral replication and cell biology have made widespread use of two sets of tools that bear comment. Proteins and natural products that bind to cholesterol, such as perfringolysin-O and fillipin, have been used to visualize the subcellular localization of cholesterol in microscopy studies. Methyl-beta-cyclodextrin has been used to deplete cells and membranes of cholesterol for loss-of-function studies. A limitation of these studies is that these reagents are not selective for cholesterol and bind to many other late-stage sterols. This selectivity (or lack thereof) should be recognized when designing and interpreting these types of experiments. For example, the loss of function caused by depletion of cholesterol with methyl-beta-cyclodextrin is commonly "rescued" by the addition of exogenous cholesterol without the inclusion of other sterols as controls. This overlooks the significant differences in the effects of cholesterol versus other sterols on the functional properties of membranes (Bloch, 1983). Likewise, the use of fluorophore-conjugated sterols in imaging experiments carries the risk that the fluorophore, which is generally comparable in size to the sterol moiety, affects the physical and/or chemical properties of the molecule.

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or endosomal membranes (togaviruses). Regardless of the donor membrane, the replication compartments characterized to date can generally be categorized into one of two classes: invaginated vesicles or double-membrane vesicles (Paul and Bartenschlager, 2013) (Figure 3). Viruses such as the alphaviruses and flaviviruses replicate within single-membrane invaginations that are continuous with the donor membrane and connected to the cytosol through a "neck-like" structure. Viruses such as HCV, poliovirus, coxsackie B3 viruses, and SARS-coronavirus replicate their genomes within double-membrane vesicular structures that are connected to one another through their shared outer membrane. Membrane invagination requires negative membrane curvature, with bending of the membrane away from the cytoplasm, whereas double-membrane vesicles exhibit positive membrane curvature, with the membrane bending toward the cytoplasm. Membranes at the neck-like structure require membrane curvature of the opposite polarity. Formation of both types of replication compartments requires remodeling of the donor membrane through interaction with proteins or protein complexes as well as through alteration of the lipid content of the membrane.

3.2.2 Perturbation of Lipid Biosynthesis and Trafficking by RNA Viruses

Positive-strand RNA viruses perturb lipid biosynthesis and trafficking to facilitate formation and maintenance of the subcellular compartments where RNA replication occurs. The enteroviruses alter sterol trafficking and metabolism, and RNA replication for these viruses is sensitive to experimental conditions that inhibit cholesterol trafficking and/or deplete intracellular cholesterol (Box 3). The dengue virus NS3 protein directly recruits fatty acid synthase (FAS) to the replication compartment, and the resulting de novo synthesis of fatty acids is thought to enhance membrane fluidity and support membrane biogenesis at the site of RNA replication (Heaton et al., 2010). Although the molecular structures of the fatty acids produced have not been elucidated, activation of FAS enzymatic activity by NS3 reinforces the idea that lipids induced by the virus are directly involved in replication of the viral genome. Targeted profiling of the whole-cell lipidome of dengue virus-infected mosquito cells (Perera et al., 2012) has revealed increases in the steady-state abundance of unsaturated phospholipids, sphingomyelin,
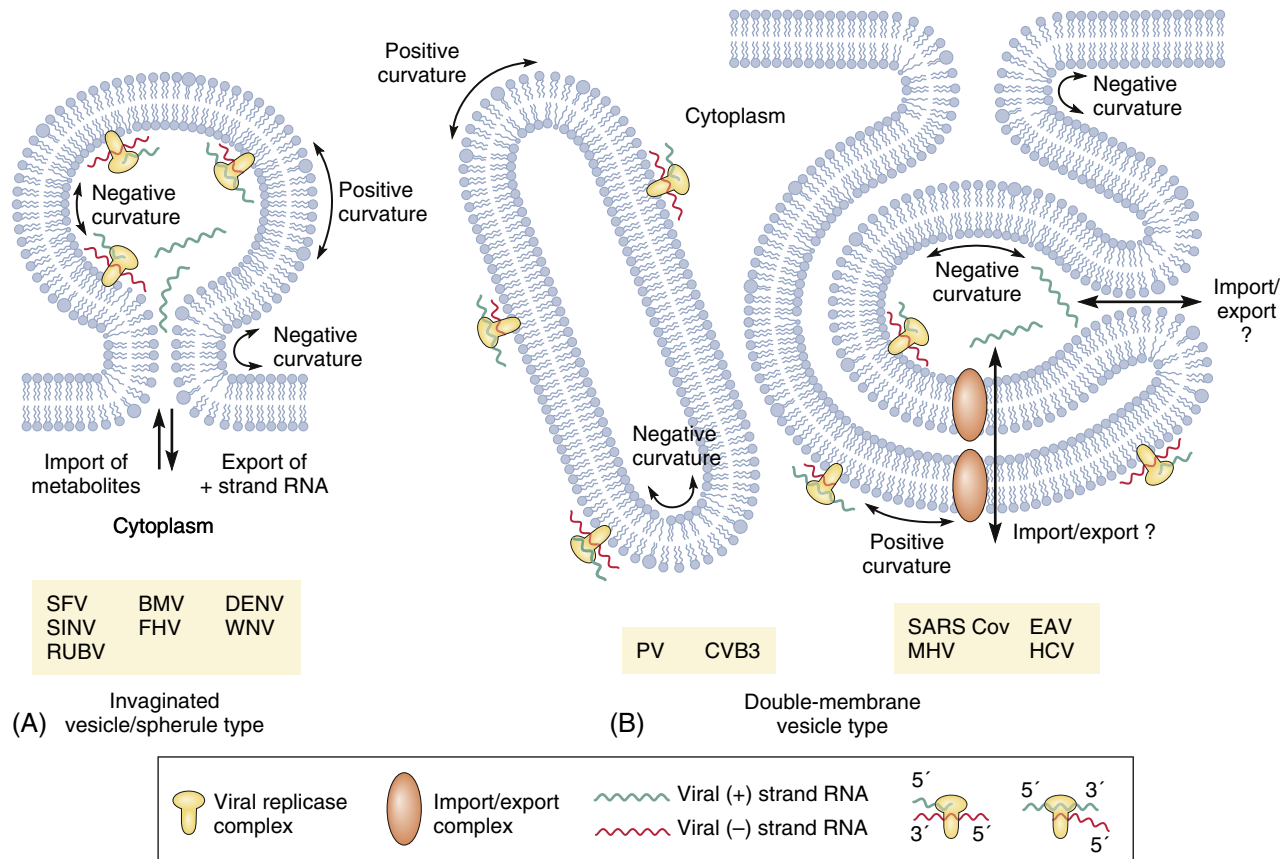
FIGURE 3 Membrane curvature in RNA virus genome replication membranes. (A) Membrane curvature is required to form invaginated vesicles. Negative curvature in the interior of the replication compartment, where RNA synthesis occurs, changes polarity at the neck structure that connects to the cytosol. Invaginated vesicle compartments are used for genome replication by Semliki Forrest virus (SFV), Sindbis virus (SINV), Rubella Virus (RUBV), Brome mosaic virus (BMV), Flock house virus (FHV), Dengue virus (DENV), and West Nile virus (WNV). (B) Double-membrane vesicle (DMV) RNA replication compartments have membrane topologies that also require the induction of negative and positive curvature although details of membrane topology and the site of RNA synthesis are still being defined. Two possibilities are illustrated. (*left*) The genome replication of enteroviruses, such as poliovirus (PV) and Coxsackievirus B3 (CVB3), is believed to occur on the outer membrane of single-membrane tubules and DMVs (inner membrane is not depicted for simplicity). Positive curvature on the exterior of the DMV is likely promoted by the presence of PI4P lipids, which have also been shown to recruit the 3D^{pol} of poliovirus (Figure 4). (*right*) Ultrastructural studies suggest that viruses such as hepatitis C virus (HCV), severe acute respiratory syndrome coronavirus (SARS-CoV), mouse hepatitis virus (MHV), and equine aterivirus (EAV) perform RNA replication in the lumen of the DMV, although it is less clear if an opening connects the interior of the DMV with the cytosol and/or if specific transporters facilitate import and export from this compartment. *From Paul and Bartenschlager (2013).*

and ceramide as well as diacylglycerol and phosphatidic acid. Biochemically isolated replication membranes exhibit enriched ceramide content, including specific enrichment of long-chain ceramide and dihydroceramide species that may induce negative curvature to facilitate invagination of the endoplasmic reticulum membrane and formation of the replication compartment. This is consistent with the known effects of ceramides on lipid bilayer structure, although it is also possible that these ceramides are generated as part of the host response or have some other function.

The replication membranes of enteroviruses, poliovirus and coxsackie B virus, and HCV are enriched in phosphatidylino-sitol-4-phosphate (PI4P) lipids, which in uninfected cells are synthesized by phosphatidylinositol 4-kinases in *trans*-Golgi-network membranes (Altan-Bonnet and Balla, 2012)

(Figure 4). For the picornaviruses, the viral 3A protein recruits PI4KIIIβ (but not PI4KIIIα) to replication membranes by modulating the activity of the Arf1/GBF1 complex and also by either a direct or indirect interaction with the kinase. HCV enriches its replication membrane with PI4P through recruit-ment and activation of PI4KIIIα through an interaction with onstructural protein 5A (NS5A). In the absence of PI4KIIIα, HCV appears to use PI4KIIIβ, although the mechanism whereby HCV affects this enzyme is less clear.

What are the functional consequences of enrichment of PI4P lipids in viral replication compartment membranes? The presence of PI4P lipids appears to be required for viral RNA synthesis since steady-state RNA replication is inhibited in the presence of small-molecule inhibitors of PI4KIII activity or when the kinase is depleted through RNAi. Consistent
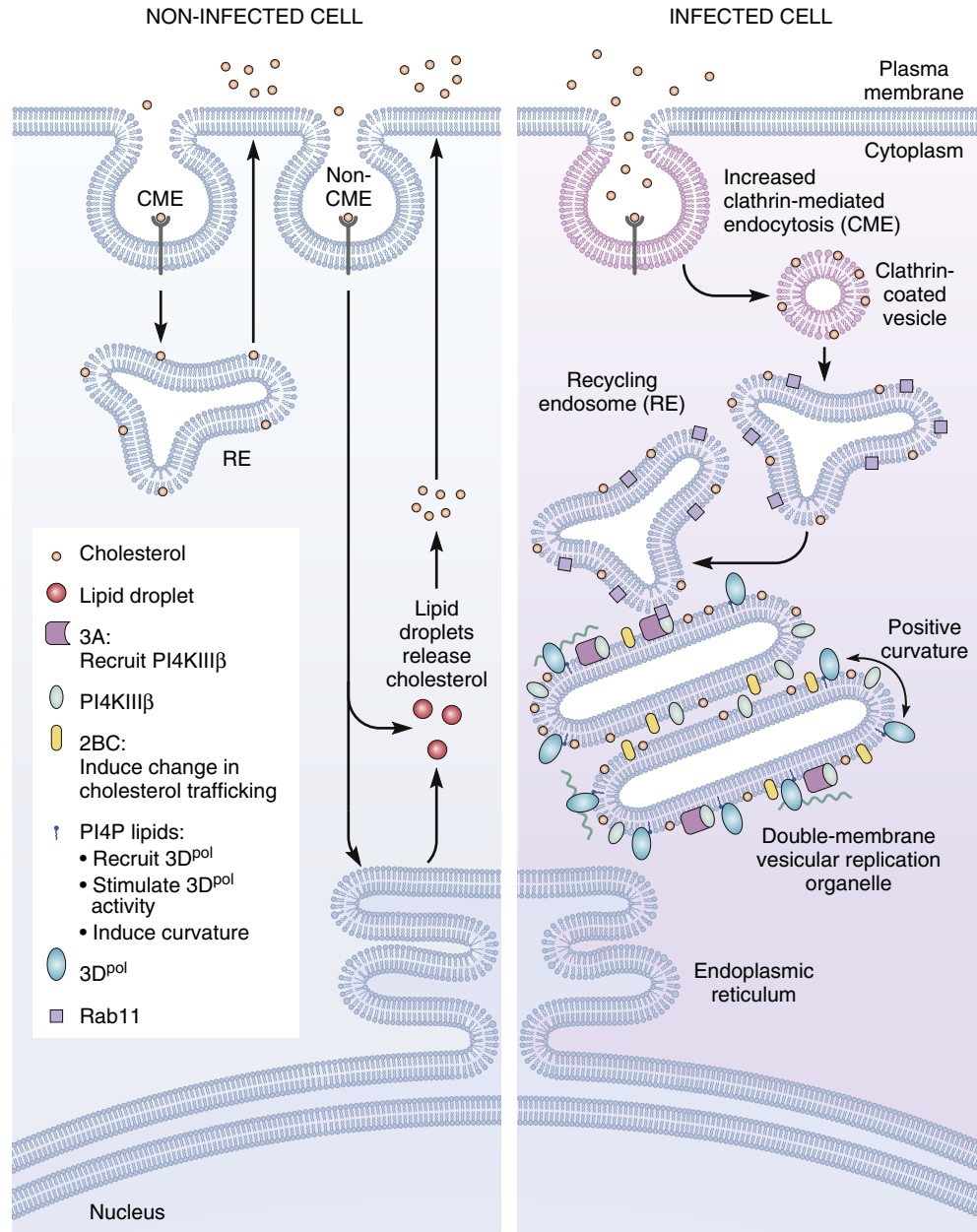
FIGURE 4 Perturbations of lipid metabolism by enteroviruses. Enteroviruses replicate on cholesterol-rich double-membrane vesicle-type (DMV-type) compartments formed from remodeling of the endoplasmic reticulum (ER), Golgi intermediate compartment (ERGIC), and Golgi apparatus. The inner membrane of the DMV is not depicted for visual simplicity. The viral 2BC protein enhances clathrin-mediated endocytosis (CME) and uptake of cholesterol, which is trafficked via recycling endosomes (RE) to the replication organelle. Direct or indirect recruitment of phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) by the viral 3A protein leads to synthesis of phosphatidyl-4-phosphate (PI4P) lipids in the replication membrane and also facilitates delivery of RE to the replication organelle via interaction of PI4KIIIβ with Rab11. A reduction in lipid droplets rich in sterol esters suggests that enteroviruses may also reduce formation of these lipid droplets and/or enhance release of sterols stored therein. *From Strating et al. (2013).*

with this, depletion of PI4P lipids reduces RNA synthesis in a cell-free model of poliovirus replication. Although the molecular basis for this effect on genome replication is still very much an open area of investigation, the cellular function of PI4P lipids is to recruit proteins to the Golgi membrane and other specific membranes by interaction with pleckstrin (PH) or epsin N-terminal homology domains. By analogy, the presence of PI4P lipids may recruit necessary components to the site of RNA replication. In support of this notion, the enterovirus RNA-dependent RNA polymerases have specific binding sites for PIP4, which may promote their recruitment from the cytosol to the site of replication.

A second possibility is that PI4P lipids induce the curvature needed to form the compartment where genome replication occurs. This could be a direct effect of PI4P structure on the membrane or an indirect effect resulting from recruitment of curvature-inducing PI4P-binding proteins, such as EpsinR and four-phosphate adaptor protein 2 (FAPP2). Although much remains to be learned about the function of PI4P lipids in positive-strand RNA virus replication, the requirement for this subset of phosphatidylinositol lipids over the other six phosphatidylinositol classes suggests a specific relationship between PI4P structure and function in viral RNA replication.

3.3 Lipids in Viral Assembly and Release

All enveloped viruses have viral membranes derived from the host cell, and the composition of the viral envelope has long been thought to provide information about the assembly and budding process. Assembly is initiated by trafficking of the viral genome and structural proteins to the host membrane where budding will occur. Budding itself requires the induction of positive membrane curvature as the nascent, enveloped virion is formed and negative membrane curvature at the neck connecting the nascent particle to the membrane from which it buds. Scission of the neck to sever the viral particle from the budding membrane completes the budding process. Viruses use diverse cellular membranes and mechanisms of varying complexity for these processes.

3.3.1 Recruitment of Viral Components to the Site of Assembly

Although inner viral structural proteins do not typically have membrane-spanning domains, they must somehow be recruited to the site of assembly and budding. For viruses such as HIV and influenza virus, which bud from raft-like microdomains at the plasma membrane, virion components undergo lipid-mediated co-sorting into the same membrane microdomain, which then serves as a platform for assembly and budding of new virions. In the case of HIV, Gag is initially recruited to the plasma membrane through its interaction with phosphatidylinositol (4,5) bisphosphate. Subsequent budding of virions requires both cholesterol and sphingolipids, consistent with a dependence on lipid-ordered domains to facilitate assembly of individual components into virions. Although other host factors contribute to these processes, the lipids involved appear to serve specific functions in spatial-temporal regulation of HIV assembly.

Viruses that bud at intracellular membranes must also concentrate virion components at sites of assembly. The host lipids and proteins used to direct this process are in some cases suspected but largely not known. HCV provides an apt illustration (Lindenbach and Rice, 2013; Filipe and McLauchlan, 2015). HCV core protein initially traffics to the surface of cytosolic lipid droplets (cLDs) where it interacts with the NS5A protein. Delivery of core and NS5A to cLDs early in infection is mediated by diacylglycerol O-acetyltransferase 1 (DGAT1), which catalyzes synthesis of triglycerides and formation of lipid droplets; Rab18, which resides on LDs and regulates vesicular trafficking; and tip-interacting protein 47 (TIP47), which regulates incorporation of triacylglycerols into LDs and LD maturation. In addition, trafficking of core to cLDs requires cytosolic phospholipase A2 (cPLA2), a key effector of arachidonic acid signaling.

Later in the replication cycle, budding of virions requires that cLD-associated core and viral genomic RNA traffic to sites where the viral E1 and E2 glycoproteins have accumulated on the lumenal side of the endoplasmic reticulum membrane. This is dependent on NS5A as well as interaction of NS2 with NS3/4A. Although the mechanisms underlying these trafficking events are still poorly understood, integration of HCV assembly with LD biogenesis is thought to enable coordination of viral translation, RNA replication, and assembly through regulated trafficking of viral components to or from the sites where these processes occur. Although our understanding of these processes is currently "protein-centric," the lipid molecules in these cellular pathways are unlikely to be passive bystanders. Investigation of the structure and function of specific lipid molecules in HCV assembly is anticipated to be necessary to understand the physical mechanisms regulating these dynamic processes.

3.3.2 Mechanisms for Inducing Membrane Curvature in Budding and Scission

Budding of viral particles requires the induction of positive membrane curvature leading to formation of a nascent virion attached to the budding membrane via a narrow-diameter neck region with negative curvature. Scission of this neck region completes budding of the viral particle. Viruses use protein–lipid and lipid–lipid interactions to achieve membrane curvature during virion budding and scission (Rossman and Lamb, 2013) (Figure 5). First, membrane curvature can be "scaffolded" by viral core proteins or proteins that bind to the head groups of the lipid bilayer. In this case, energetically favorable protein–lipid interactions are used to drive curvature of the membrane. Second, the insertion of proteins into the lipid bilayer can cause "hydrophobic displacement" leading to energetically unfavorable differences in the surface areas of the inner and outer membrane leaflets. Membrane curvature is induced in these cases to relieve the energy strain. Third, adjacent areas of lipid-ordered and lipid-disordered regions of a membrane can differ in thickness, resulting in energetically unfavorable interactions (referred to as "line tension") due to alignment of the polar head groups of the thinner lipid-disordered domain with the hydrophobic tails
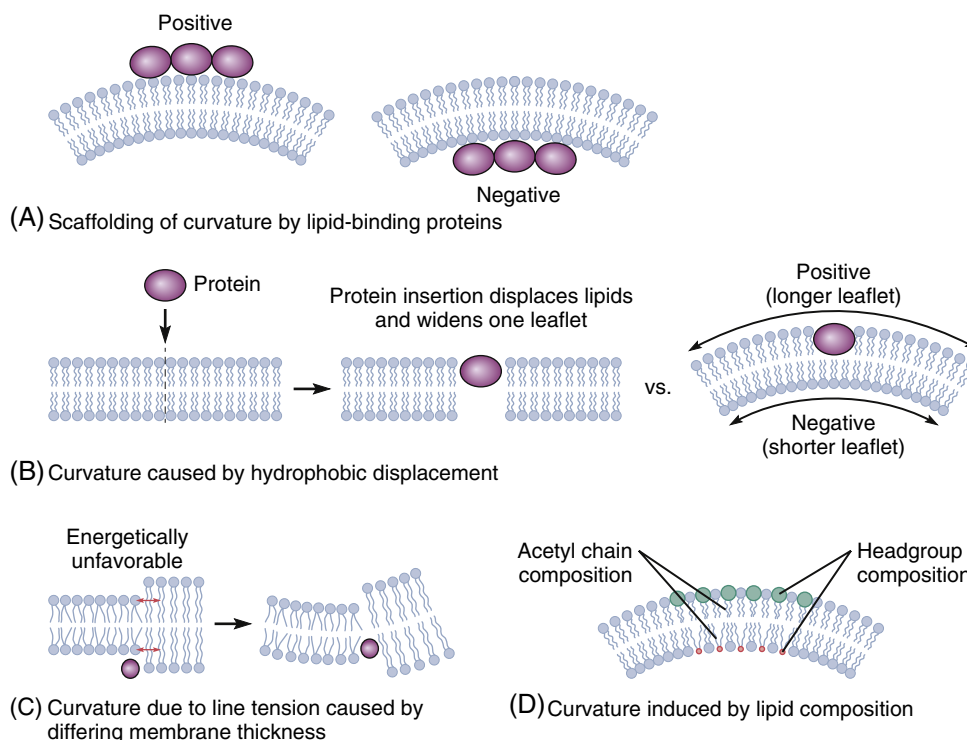
FIGURE 5 Mechanisms of inducing membrane curvature. (A) The interaction of membrane lipids with viral core proteins or other proteins that recognize lipid head groups can scaffold membrane curvature. (B) Insertion of a protein into the membrane causes asymmetric displacement of lipids. The induction of curvature is one physical mechanism for counteracting the resulting difference in surface areas of the inner and outer leaflets. (C) Adjacent regions of lipid-ordered and lipid-disordered membrane differ in thickness. This results in energetically unfavorable packing of hydrophilic head groups against hydrophobic tail groups. Membrane curvature can relieve this "line tension." (D) The structure and shape of individual lipids in the bilayer can affect membrane curvature (see Text Box 2). Cone-shaped lipids (PE) and inverse-cone-shaped lipids (PI) promote negative and positive membrane curvature, respectively.

of the thicker, lipid-ordered domain. Fourth, lipid structure can affect membrane curvature through intrinsic bending induced by the shape and charge of the component lipids (Box 2).

Perturbations of membrane curvature by viral glycoproteins on the outer leaflet of the budding membrane and matrix and capsid proteins on the inner leaflet are essential for the budding of many viruses. For viruses such as tick-borne encephalitis virus and hepatitis B virus, budding is driven by assembly of the viral glycoprotein on the surface of the nascent bud. This "pulling" force is sufficient to result in the formation and release of subviral particles that lack nucleocapsids. In contrast, for HIV and other retroviruses, the assembly of Gag exerts a "pushing force" that drives membrane curvature and is sufficient for assembly and release of virus-like particles. Membrane curvature during budding of influenza virus (see Section 3.3.3) as well as Semliki Forest virus and other alphaviruses appears to require the concerted action of pushing from the nucleocapsid and pulling by the viral glycoprotein.

Scission of the neck to release the viral particle from the budding membrane is a distinct step in viral replication, as evidenced by the detection of stalled intermediates that resemble "beads on a string" in the absence of viral proteins that recruit the scission machinery of the host cell or that catalyze membrane scission directly. Most viruses do not encode their own scission machinery, and the outer neck of the membrane requiring scission is topologically inaccessible to most cytoplasmic scission proteins (e.g., dynamin or Arf1). HIV and other retroviruses achieve scission by recruiting endosomal sorting complex required for transport (ESCRT) proteins to the site of viral assembly through late-domain motifs in HIV Gag and other retroviral capsid proteins. Other viruses (e.g., Poxviridae, Herpesviridae, Filoviridae, Rhabdoviridae, and Paramyxoviridae) that bud from a variety of subcellular membranes (nuclear, endoplasmic reticulum, or plasma membranes) also recruit the ESCRT machinery through interactions of viral late-domains with Tsg101, Alix, and Nedd4-like proteins, although there are undoubtedly variations in viral interaction with the ESCRT machinery. Viral scission can also occur via ESCRT-independent mechanisms. For example, respiratory syncytial virus has no known late-domain motifs and undergoes virus budding using a mechanism that requires Rab11-dependent pathway family-interacting protein 2 (Rab11-FIP2).

3.3.3 Influenza Virus Assembly, Budding, and Scission

Influenza virus assembly, budding, and scission present unique examples due to the extent to which these processes are mediated by viral proteins and also the extent to which they have been characterized (Martyna and Rossman, 2014). The two influenza virus glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are targeted to the plasma membrane by specific sequences in the transmembrane domains of HA and NA as well as by palmitoylation of HA. Concentration of HA in the membrane induces curvature and is sufficient to permit budding of virus-like particles; however, budding of authentic virions requires other effectors, including the viral M1 and M2 proteins. M1 multimerizes to form a helical net that supports the viral membrane and connects the inner leaflet of the viral membrane to viral ribonucleoproteins (RNPs) on the virion interior. Pushing caused by multimerization of M1 beneath the budding membrane or by recruitment of RNPs initiates budding.

Scission of the neck structure is catalyzed by the viral M2 protein although the biophysical mechanism of scission is still not known. In infected cells, M2 localizes between ordered (raft) and disordered domains of the plasma membrane. As budding progresses, M2 concentrates in the neck region at the boundary between the ordered, cholesterol-rich viral membrane and the adjacent cholesterol-poor plasma membrane. Peptides corresponding to the amphipathic helix of M2 induce negative membrane curvature in a cholesterol-dependent manner; moreover, binding of M2 to lipid domains with high radii of curvature comparable to those in the neck of budding virions disrupts packing of lipid head groups in vitro. Insertion of the M2 amphipathic helix into the lipid-disordered phase may therefore cause membrane scission through the generation of line tension or packing defects. Cellular factors (e.g., Rab11) may also contribute to scission since mutations in the cytoplasmic tail of M2 can have significant effects on budding efficiency.

3.4 Lipids in the Inflammatory Response

The host response to viral infection in many cases contributes to viral pathogenesis through the overabundant production of cytokines (termed a "cytokine storm") and damage caused by inflammatory cells of the host response. Autocrine and paracrine signal transduction by bioactive lipids is central to both the activation and resolution of the host inflammatory response. These lipids are synthesized from arachidonic acid and other polyunsaturated fatty acids (e.g., eicosapentaenoic, docosahexaenoic, linoleic, and linolenic acids) via cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 pathway. Their release from membrane phospholipids is tightly regulated by a system of over 50 enzymes. COX-1 and COX-2 are responsible for synthesis of prostaglandins and thromboxanes, which generally exert a strong proinflammatory effect early in the host response. The three LOX enzymes (5-LOX, 12-LOX, and 15-LOX) synthesize leukotrienes and lipoxins as well as hepoxilins and hydroperoxy and hydroxyl fatty acids. Lipids produced by 5-LOX (LTB$_4$, LTC$_4$, LTE$_4$, 5-HETE) are proinflammatory, acting as chemoattractants for neutrophils and basophils and potently promoting bronchoconstriction, smooth muscle contraction, and increased vascular permeability. In contrast, lipids produced by 12-LOX and 15-LOX (LXA$_4$ and LXB$_4$) have anti-inflammatory activities that are important for resolution of the inflammatory response. Resolvins, protectins, and maresins produced from eicosapentaenoic and docosahexaenoic acids are also important in limiting infiltration of immune cells and tissue damage at the site of infection and are therefore important for resolution of the inflammatory response.

As illustrated by recent studies of influenza virus, analysis of the differential induction of bioactive lipid mediators of inflammation in high- versus low-pathogenicity viral infections has been informative for understanding pathogenesis and for suggesting new strategies to counteract or prevent misregulated host responses associated with severe disease. Parallel transcriptomic, proteomic, and targeted lipidomic profiling experiments have been used to identify differences in the induction of bioactive lipids mediating the host inflammatory response to high and low pathogenicity strains of influenza virus. Targeted profiling of broncho-alveolar lavage samples from mice infected with the pathogenic mouse-adapted PR8/H1N1 virus, or lethal or sublethal doses of low pathogenicity X31/H3N2 virus revealed that the pathogenic phase of PR8 infection is associated with an elevation of 5-LOX proinflammatory metabolites (Tam et al., 2013). In contrast, the resolution phase of X31 infection is associated with elevated 12-LOX anti-inflammatory metabolites.

Building on these animal model studies, preliminary analysis of nasal wash samples from human influenza virus infections suggests that increased levels of 5-LOX-derived metabolites and decreased levels of 12-LOX-derived metabolites is also correlated with increased clinical symptoms and immune responses. Decreased production of metabolites from the 12/15-LOX pathway, DHA-derived protectin D1, and HDoHE in lung tissue is also correlated with high-pathogenicity viral infections (Morita et al., 2013). 12/15-LOX metabolites are thus markers of the resolution of inflammation during the host response to influenza virus, but whether they promote the resolution of the inflammatory response and protect from pathogenesis is unproven. Here, it is worth noting that Morita and colleagues identified protectin D1 as an inhibitor of influenza virus replication in human respiratory cells and found that its antiviral mechanism of action in cell culture is due to an effect on the nuclear export of viral transcripts. In the mouse model, administration of protectin D1 increases survival, but the extent to which this is due to an effect on viral replication versus mitigation of the host inflammatory response is less clear. These findings illustrate the reality that lipids and other small molecules

can affect biological systems via multiple mechanisms, which may or may not be related. Detailed molecular characterization of lipid function in these systems is necessary to deconvolute and understand these effects.

4. CONCLUDING REMARKS: EXPLOITATION OF VIRAL DEPENDENCE ON HOST LIPIDS AS A SELECTIVE ANTIVIRAL STRATEGY

The study of lipid structure and function can be viewed as a challenge for systems biology. As the functional output of the expressed genome, the lipidome and metabolome represent the integration of all virus-induced changes in host transcription, RNA stability and processing, translation, posttranslational modifications, protein stability and localization, and enzymatic activity. A global view of these perturbations and their functional consequences is important for both basic and translational research efforts. As basic science, the interaction of viruses with host lipids is a fundamental aspect of viral replication and pathogenesis. We cannot understand the physical and molecular mechanisms driving viral processes without understanding the function of membranes associated with these processes and how, at a molecular level, lipid structure dictates function. Although our knowledge of the specific lipids required for viral replication and pathogenesis is arguably still too limited, the idea of antiviral strategies that exploit the interaction of viruses with host lipids is a compelling area for translational efforts. In part, this is because Nature already provides multiple examples in which the modulation of membrane composition is an effective defense against viral pathogens. For example, the interferon-inducible transmembrane proteins (IFITMs) exhibit broad-spectrum activity against many enveloped viruses and may do so by affecting the composition or packing of cellular membranes (Bailey et al., 2014). Consistent with this, the IFITMs affect intracellular lipid transport, and their presence in membranes enhances lipid packing and reduces membrane fluidity. Likewise, the effects of 25-hydroxysterol on sterol and sphingolipid synthesis are thought to directly affect the composition of host membranes targeted for viral fusion or used for other viral processes. Rational antiviral therapies that act through analogous mechanisms are therefore theoretically possible, although significant advances are needed in our understanding of how lipids function in viral infection to realize this potential.

We currently lack understanding of how lipid structure affects lipid function in vivo. This is a largely unexplored area of virus–host interactions despite clear significance for basic science and translational efforts. Ideally, tools analogous to site-directed mutagenesis studies of protein structure and function could be used to understand how seemingly subtle differences in structure (e.g., acyl chain length and degree and location of unsaturation, ratio of glycerophospholipids to sphingolipids, or head group charge and size) can

lead to significant changes in function. In reality, this type of reductionist experiment is very challenging. Engineering advances that enable the synthesis of biomimetic-supported lipid bilayers from synthetic and cell-derived lipids in a medium-throughput manner may facilitate screens to identify specific lipids or combinations of lipids that are optimal for interaction with viral proteins. Viral processes, such as membrane fusion or penetration during entry or genome replication, may also be amenable to functional screens of this type that probe the relationship between lipid structure and membrane function in the associated virological process.

With respect to lipidomic profiling, current NMR and LC-MS-based approaches only permit the examination of lipid structure and abundance in biological samples derived from populations of cells. Determining the subcellular localization of lipids requires purification of the membrane of interest away from other cellular membranes or microscopy methods using fluorophore-conjugated lipid molecules or specific lipid-binding proteins to detect the lipid of interest. These approaches may be limited, respectively, because the membrane of interest cannot be biochemically isolated without perturbing it, because attachment of a fluorophore alters the physicochemical properties of the lipid molecule, and because the lipid-binding protein used for detection lacks sufficient specificity (Box 3). Alternative approaches that overcome these technical limitations are clearly needed, and emerging imaging methods may have a transformative effect on the study of lipids in viral processes in the cellular milieu. Raman-based microscopy methods rely on the vibrational frequency of chemical bonds and are especially well-suited to the imaging of lipids since they are rich in carbon-hydrogen bonds. Coherent Raman microscopy has been used to demonstrate variations in the degree of acyl chain saturation and order among droplets within the same cell and within the same droplet. This approach coupled with confocal fluorescence microscopy to detect viral proteins could be developed to study the chemical content of membranes associated with specific viral processes and to monitor the reactions that result in synthesis of these membranes in live cells. Here, isotopically labeled lipids of interest could be especially useful, since the imaging system can be tuned to detect only the isotopically labeled chemical bond and this could be used to distinguish a particular lipid species from other members of its class (e.g., visualization of deuterated desmosterol without background signal from endogenous cholesterol and other sterols). Imaging MS methods may also prove useful for monitoring lipid localization within the cell. Methods such as nanoscale secondary ion MS and desorption electrospray ionization MS can resolve the localization of a given metabolite in two-dimensional space and have been used to monitor lipid droplet formation in cells and tissues and may, with gains in spatial resolution and sensitivity, provide another tool for analysis of membrane-associated viral processes in cells.

As with all host-targeted antivirals, a crux issue is whether it is possible to inhibit viral replication or pathogenesis by targeting lipid metabolism without having undesired effects on

the host. This concern arises especially because metabolism is central to host homeostasis and viability. The identification of lipid molecules that are required for viral replication but dispensable for the host, may present the best option for mitigating the risk of toxicity at the cellular or organismal level. Untargeted lipidomic profiling experiments and metabolomic flux studies are critical tools for this effort. The tools and approaches used to advance our understanding of how specific lipid molecules function in viral processes may also facilitate rational targeting of specific lipid molecules rather than wholesale blockade of entire biosynthetic pathways. This may enable minimization of toxicity and maximization of tolerability and safety. Rapid advances in methodology and understanding make this an exciting and rapidly evolving area of virology and systems biology.

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