

Maturation of the Gag core decreases the stability of retroviral lipid membranes

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ABSTRACT

To better understand how detergents disrupt enveloped viruses, we monitored the biophysical stability of murine leukemia virus (MLV) virus-like particles (VLPs) against a panel of commonly used detergents using real-time biosensor measurements. Although exposure to many detergents, such as Triton X-100 and Empigen, results in lysis of VLP membranes, VLPs appeared resistant to complete membrane lysis by a significant number of detergents, including Tween 20, Tween 80, Lubrol, and Saponin. VLPs maintained their structural integrity after exposure to Tween 20 at concentrations up to 500-fold above its CMC. Remarkably, VLPs containing immature cores composed of unprocessed (uncleaved) Gag polyprotein were significantly more resistant to detergent lysis than VLPs with mature cores. Although the maturity of retroviral Gag is known to influence the stability of the protein core structure itself, our studies suggest that the maturity of the Gag core also influences the stability of the lipid bilayer surrounding the core.

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Introduction

Enveloped viruses are characterized by the encapsulation of viral proteins and genetic elements by a lipid membrane acquired directly from the host cell during viral budding. While some enveloped viruses bud from cellular compartments such as the endoplasmic reticulum (Mukhopadhyay et al., 2005) or the nucleus (Mettenleiter et al., 2006), many, including murine leukemia virus (MLV) and human immunodeficiency virus (HIV), usually bud from the cellular plasma membrane (Cadd et al., 1997; Pelchen-Matthews et al., 2003; Pornillos et al., 2002; Sherer et al., 2003). By directing the cellular location of budding, viral structural proteins, such as the matrix component of the retrovirus Gag polyprotein, help to determine the lipid composition of the viral membrane (Ono et al., 2000; Spearman et al., 1997; Zhou and Resh, 1996). Many enveloped viruses, including MLV and HIV, are believed to selectively target sub-fragments of the plasma membrane known as lipid rafts (or detergent-resistant membranes, DRMs, as they are known when isolated) (Nguyen and Hildreth, 2000; Ono and Freed, 2001; Pickl et al., 2001; Suomalainen, 2002). Budding from lipid rafts enriches the membranes of these viruses with cholesterol, sphingomyelin, and

gangliosides (Aloia et al., 1988, 1993; Brugger et al., 2006), which are known to stabilize lipid structures.

The lipid composition of viral membranes is a critical determinant of viral stability and can influence the potential for viral resilience and transmission (Aloia et al., 1988, 1993; Brugger et al., 2006). Detergents are commonly employed for sanitizing surfaces and fluids against enveloped viruses, and previous studies demonstrate the efficacy of detergents against infectivity of enveloped viruses (Horowitz et al., 1998; Oxford et al., 1971). However, these types of studies commonly use detergent concentrations substantially above the detergent CMC and generally are not focused on understanding their mechanism of inhibition. On the basis of their disruptive activity in other membrane types, it has largely been assumed that detergents act as lytic agents on the viral membrane. However, the biophysical effects of different detergents on viral lipid membrane structures have not been directly investigated. The lack of such knowledge is significant given the common use of detergents in research and clinical protocols. For example, many protocols used for blood products employ Tween detergents to inactivate enveloped viruses (Chernomordik et al., 1995; De Tkaczewski et al., 1968; Roberts, 2000; Seitz et al., 2002; Stromberg, 1972; Webster and Darlington, 1969). From a therapeutic perspective, viral membranes represent potential targets for the activity of compounds that demonstrate selective activity compared to host membranes, and two small molecule compounds with preferential activity against membrane enveloped viruses have been described (St Vincent et al., 2010;

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Wolf et al., 2010). Although the biochemical compositions of many viral lipid membranes are well characterized, elucidating the biophysical properties of viral membrane structures may enable more effective mechanisms of viral control and generate new insights into mechanisms for selectively disrupting viral membranes.

To understand how detergents affect enveloped virions, we tested a panel of commonly used detergents against murine leukemia virus (MLV) virus-like particles (VLPs) using real-time (optical biosensor) and conventional measurements of viral lipid membrane integrity. Our results confirm that exposure to many detergents results in lysis of VLP membranes. However, MLV VLPs appeared highly resistant to lysis by a number of other detergents, including those recommended for clinical use. Most surprisingly, VLPs containing immature cores composed of unprocessed (uncleaved) Gag polyprotein were significantly more resistant to detergent lysis than VLPs with mature cores. The profiles of membrane lysis suggest that the core protein structure determines whether the lipid bilayer is completely disrupted and dissociates from Gag. Although the maturity of retroviral Gag is known to influence the stability of the protein core structure itself, our studies suggest that maturation of the Gag core also decreases the stability of the lipid bilayer surrounding the core.

Results

Detection of viral lipid membrane rupture by detergents

We previously showed that MLV virus-like particles (VLPs) can be efficiently captured onto biosensor surfaces using antibodies directed against receptors expressed on the surface of virus membranes (Willis et al., 2008). Using this approach,

we developed a real-time optical biosensor rupture assay on captured VLPs exposed to a panel of detergents. The purified VLPs used in these experiments are approximately 150 nm in diameter in size and substantially free of contaminating cellular membranes, as determined by peak measurements using dynamic light scattering (Sup. Fig. 1). Purified MLV VLPs were efficiently captured on a Biacore biosensor chip using an antibody (JS-81) directed against the cell-surface protein CD81 (a schematic of the capture event is depicted in Fig. 1A). CD81 is a member of the tetraspanin family of proteins (containing four transmembrane domains) and was chosen as a target for capture because it is endogenously expressed in HEK-293 cells and is incorporated and evenly distributed onto MLV VLP surfaces during production (at least 16 copies of CD81 per virion) (Segura et al., 2008).

Since biosensor surface plasmon resonance (SPR, measured in resonance units, RU) is directly proportional to the mass of molecules on the chip surface (Canziani et al., 1999; Wang et al., 2010) (within ~200 nm distance from the chip surface), the amount of VLPs remaining attached to the surface could be directly monitored in real-time while increasing concentrations of detergent were flowed across (a complete experiment is depicted in Fig. 1B). The constant flow of detergent over the experimental substrate represents a distinct advantage of this methodology because the detergent concentration is maintained as a constant throughout the experiment due to its continual renewal. In comparison, traditional fixed-volume detergent exposure experiments (e.g. in cuvettes or wells) result in a reduction in free detergent concentration upon detergent binding events, which can be significant in cases where low concentrations of detergent are employed. A sensorgram obtained in the absence of VLPs shows no change in baseline signal other than the transient spikes

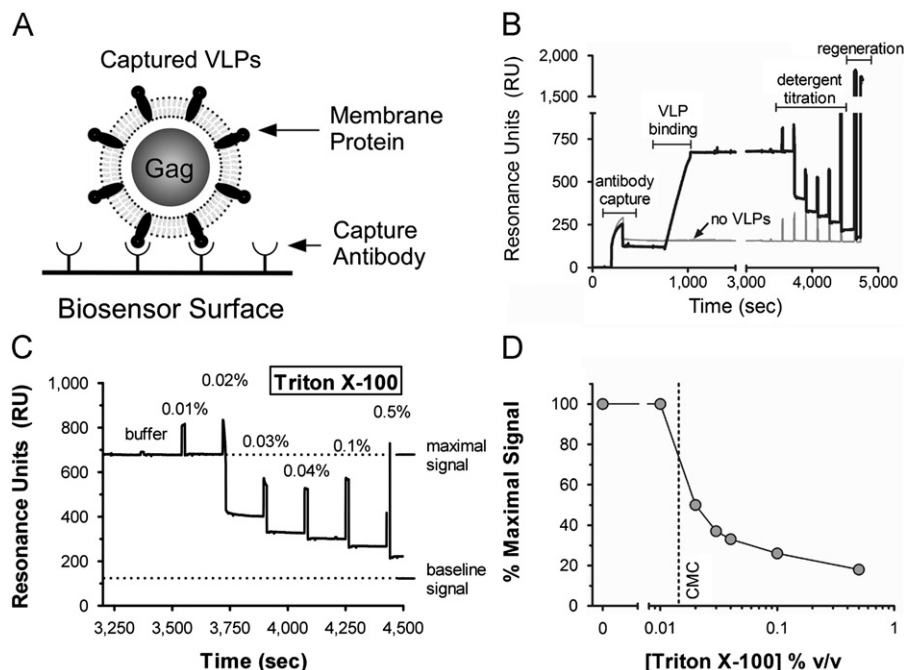


Fig. 1. Detection of detergent-mediated rupture of viral membranes using an optical biosensor. (A) Schematic representation of VLP association with a biosensor chip via specific capture antibodies. (B) Sample sensorgram for a complete biosensor VLP rupture experiment. Binding of capture antibody and VLPs results in increased optical resonance units (RU) on the biosensor chip surface. Upon titration with increasing concentrations of detergent for 15 s (Triton X-100 shown), decreases in VLP mass are detected when VLPs rupture and mass dissociates from the biosensor surface. Transient spikes in the sensorgram are due to refractive index changes upon detergent injections. A sensorgram obtained for a control experiment in the absence of VLPs (gray trace) shows no change in signal other than the transient spikes associated with detergent injections. The biosensor chip is regenerated to the anti-mouse Fc covalently coupled to the surface prior to re-use. (C) The detergent titration region of the sensorgram trace is shown in greater detail to demonstrate the effect of increasing concentrations of Triton X-100 on VLPs bound to the chip surface. Baseline signal in the absence of VLP binding is denoted by a dotted line. (D) To quantify the effect of detergent on VLP membrane integrity, the biosensor signal (RU) is plotted against detergent concentration. RU values are baseline subtracted and expressed as a percent of total RU of captured VLPs in the absence of detergent. The CMC of Triton X-100 is indicated with a vertical dotted line.

associated with detergent injections, demonstrating that the loss of VLP mass is not the result of dissociation of the capture antibody from the biosensor.

The critical micelle concentration (CMC) of a detergent is the concentration of detergent where it begins forming micelles. We therefore expected that at approximately the CMC of each detergent, lipid-detergent micelles would begin forming, stripping lipids out of the viral membrane and eventually causing complete disruption of the lipid membrane and release of the viral Gag proteins from the chip surface (a representative sensor-gram is depicted in Fig. 1C). Thus, as expected, at approximately the CMC of Triton X-100 (Fig. 1D, dashed vertical line), rupture of the viral membrane and loss of the bulk of the virion mass was observed as a dramatic decrease in surface plasmon resonance RU. Nearly all of the virion mass was eliminated from the chip within about a 10-fold concentration above the CMC, indicative of complete rupture of the viral particles. Triton X-100 below its CMC had little or no effect on viral structural integrity. These results demonstrate that real-time measurement of mass changes by optical biosensor can be used to detect viral membrane rupture.

Resistance of VLP membrane rupture to several detergents

We next used the optical biosensor rupture assay to investigate the effects of a panel of commonly used detergents on mature VLP membrane stability. Triton X-100, β -octylglucoside (BOG), CHAPS, NP-40, and Empigen all disrupted viral membranes at concentrations close to their CMC, as expected for most detergents interacting with lipid membranes (Helenius and Simons, 1975; Schuck et al., 2003) (Fig. 2A–E). In contrast, we were surprised to find that other detergents, including Tween 20, Tween 80, Lubrol, and saponin, caused minimal or no rupture of VLPs, even at concentrations exceeding their CMC by 500-fold (Fig. 2F–I). For example, VLPs maintained their structural integrity after exposure to Tween 20, a detergent recommended for clinical viral inactivation (Chernomordik et al., 1995; De Tkaczewski et al., 1968; Roberts, 2000; Seitz et al., 2002; Stromberg, 1972; Webster and Darlington, 1969), at concentrations up to 5%. To demonstrate that VLP rupture was not dependent on the capture antibody or protein tether used, we also tested VLPs containing the seven-transmembrane GPCR CXCR4. Capture of CXCR4 VLPs using the MAbs 12G5, which recognizes a conformationally-sensitive epitope on CXCR4 (Babcock et al., 2001; Endres et al., 1996), results in nearly identical rupture profiles. The concentration at which the initial loss of VLP mass was observed (its ‘rupture point’) was quantified for each detergent (Table 1).

Uncleaved Gag stabilizes the viral lipid membrane

The MLV structural core is composed of the Gag polyprotein and is associated with the internal leaflet of the lipid membrane through the matrix (MA) component via N-terminal myristoylation. During viral maturation, MA is proteolytically cleaved from the other subcomponents of Gag (e.g. capsid, nucleocapsid) by a viral protease. Immature Gag proteins that do not undergo proteolytic cleavage remain tethered to the inner viral membrane as an intact polyprotein. Isolated immature Gag core structures are known to be more resistant to detergents than mature cores (Kol et al., 2006, 2007; Stewart et al., 1990). However, the lipid bilayers surrounding immature and mature cores are biochemically identical so have largely been assumed to be structurally identical and have similar detergent sensitivities.

To investigate whether maturity of the viral core affects lipid membrane sensitivity to detergents, we produced MLV VLPs containing an immature (uncleaved, pol deficient) Gag structural

core (‘Gag-His’, Fig. 2). Immature VLPs were attached to Biacore biosensor chips and their structural integrity monitored during detergent exposure. Surprisingly, the mass of immature VLPs released from the biosensor surface upon exposure to most lytic detergents was significantly reduced compared to mature VLPs (Fig. 2A–D). Similar rupture profiles were observed independent of the capture antibody or protein tether used (i.e. JS-81 against CD81 or 12G5 against CXCR4). Even very high concentrations of Triton X-100 (up to 5%) failed to completely remove immature VLPs from the chip surface. One exception to these results was Empigen detergent treatment, which ruptured the membranes of both mature and immature VLPs to an equal extent (Fig. 2E). Both mature and immature VLPs were resistant to the non-lytic detergents such as Tween 20, Tween 80, Lubrol, and saponin (Fig. 2F–I). Identical sensitivities were obtained using VLPs engineered to contain a different immature Gag polyprotein comprising a fusion protein (Gag-GFP), demonstrating that these results are not due to non-specific effects of the particular Gag construct used. These results suggest that the retroviral lipid bilayer is less susceptible to complete lysis and dissociation when surrounding an unprocessed, immature viral core.

VLPs containing uncleaved Gag are stable with prolonged exposure to Triton X-100

The stability of VLP membranes in the presence of several detergents, even at concentrations known to eliminate viral infectivity, was surprising. To test whether the resistance of VLPs to certain detergents was due to insufficient exposure time, biosensor experiments were repeated for Tween 20 and Triton X-100 using prolonged detergent exposure times. Our results indicate that Tween 20 could dissociate virions from the surface to a limited extent, but only for mature VLPs, at very high concentrations (5% Tween 20 is > 700 fold above its CMC), and in combination with prolonged exposure times (15 min under continuous flow conditions) (Fig. 3A). Even under these conditions, however, the loss of signal from the biosensor surface appeared gradual and unrelated to the CMC of Tween 20, unlike the abrupt signal changes seen for lytic detergents such as Triton X-100 upon crossing CMC thresholds. Importantly, prolonged exposure of immature VLPs (Fig. 3B) to either Triton X-100 or Tween 20 failed to remove any additional VLP mass from the surface (unlike mature VLPs), consistent with the greater stability of immature VLPs observed in response to diverse detergents. The rupture point (concentration of first loss of mass) upon exposure to Triton X-100 remained identical between mature and immature VLPs regardless of the flow time, suggesting that the forces removing the VLPs at the detergent CMC (rupture) are different than the forces dissociating the VLPs over time. These results suggest that viral membranes surrounding immature Gag cores are resistant to some detergents even with prolonged detergent exposure times.

Immature Gag protein is selectively retained upon treatment of VLPs with Triton X-100 detergent

While biosensor measurements provide valuable real-time detection of mass changes, we wanted to confirm these results using an independent measure of viral membrane rupture. To do this, we assayed for the presence of Gag protein by western blot following treatment of VLPs with detergents (Fig. 4). Immature or mature VLPs were captured onto an ELISA plate, treated with 1% Tween 20, 1% Triton X-100, or 1% Empigen, and washed to remove ruptured membranes and released Gag protein. The remaining contents of each well were harvested, run on SDS-PAGE gels, and assayed for retention of Gag protein (indicative of VLP stability in

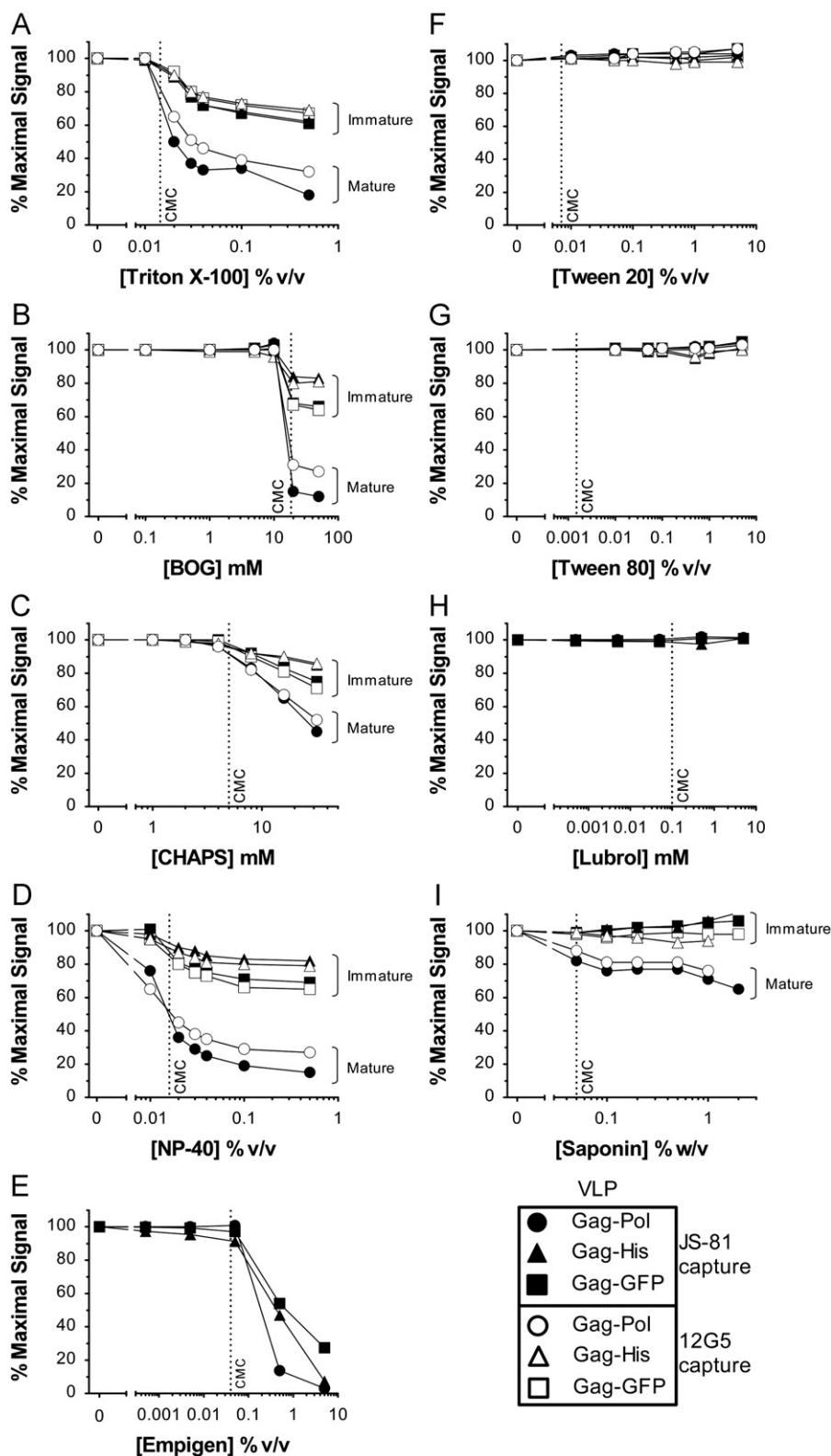


Fig. 2. Resistance profiles of VLP membranes to a panel of diverse detergents. Mature VLPs (Gag-Pol, circles) and two different types of immature VLPs (Gag-His, triangles and Gag-GFP, squares) were captured onto a Biacore C1 chip followed by treatment with increasing concentrations of the indicated detergents for 15 s. To control for the use of capture antibody, VLPs were captured using an antibody against either endogenous CD81 (JS-81, filled symbols) or against the GPCR CXCR4 (12G5, open symbols). Optical resonance signals were normalized to the maximum signal obtained using untreated VLPs. The CMC of each detergent is indicated with a vertical dotted line. $n=3$, representative plots from a single experiment are shown.

the presence of detergent) by western blot using a Gag-specific polyclonal antibody. We found that immature VLPs washed with Tween 20 or Triton X-100 retain uncleaved Gag protein at

comparable levels to mock treated VLPs (Fig. 4, left panel), consistent with their greater stability observed by biosensor measurements. In contrast, mature VLPs retained Gag proteins

Table 1
Critical micelle concentrations and VLP rupture points for detergents.

Detergent	Class	CMC	Rupture point ^a
Triton X-100	Non-ionic	0.2–0.3 mM (0.0144% v/v)	0.02% v/v
β-octylglucoside (BOG)	Non-ionic	14.5–25 mM	20 mM
CHAPS	Zwitterionic	4–6 mM	8 mM
NP-40	Non-ionic	0.29 mM (0.016% v/v)	0.02% v/v
Empigen BB	Zwitterionic	1.6–2.1 mM (0.04% v/v)	0.05–0.5% v/v ^b
Tween 20	Non-ionic	0.06 mM (0.007% v/v)	No rupture
Tween 80	Non-ionic	0.012 mM (0.0015% v/v)	No rupture
Lubrol	Non-ionic	0.1 mM	No rupture
Saponin	Porator	0.05% w/v	0.05% w/v

^a The experimental rupture point of mature VLPs is defined by the concentration of detergent that caused the first loss of mass from the biosensor surface.

^b The rupture point for Empigen is reported as a range to more accurately reflect its actual rupture point. VLPs were exposed to 0.05% and 0.5% Empigen (a 10-fold range), whereas the reported CMC of Empigen is 0.04% (Fig. 2E).

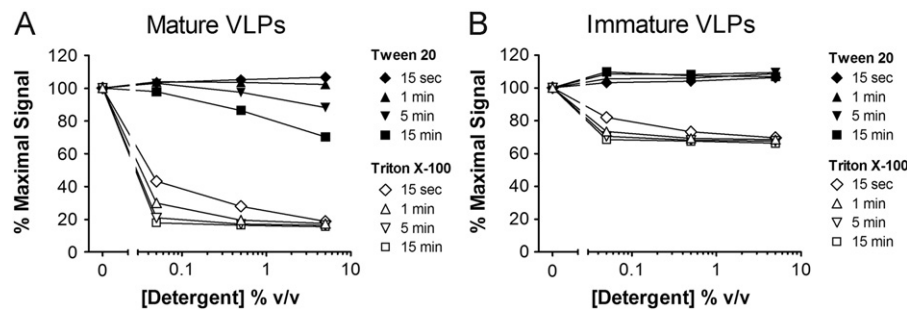


Fig. 3. Prolonged exposure of VLP membranes to Triton X-100 or Tween 20. (A) Mature or (B) immature VLPs were captured onto a Biacore biosensor chip and exposed to increasing concentrations of Tween 20 or Triton X-100 up to 5% v/v. Detergents were flowed over VLPs for a duration of 15 s, 1 min, 5 min, or 15 min to assess the effect of prolonged detergent exposure time on VLP stability. The optical resonance signals were normalized to the maximum signal obtained using untreated VLPs. $n=3$, representative plots from a single experiment are shown.

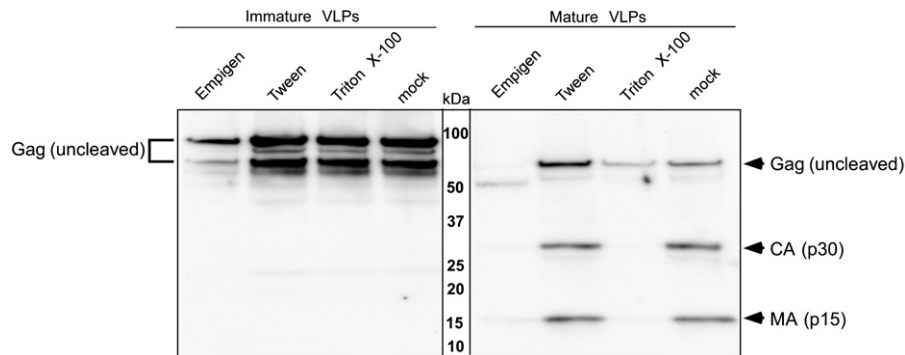


Fig. 4. Gag retention in immature and mature VLPs following detergent treatment. Immature (Gag-GFP) and mature VLPs (Gag-Pol) were captured on a 96-well ELISA plate using WGA lectin, followed by incubation with either Tween 20, Triton X-100, Empigen (all at 1%), or a no detergent control (mock). VLPs were washed three times, and the remaining contents of each well were resuspended in SDS-PAGE loading buffer. Samples were run on a 4–20% SDS PAGE gradient gel, transferred to PVDF, and probed with a Gag specific polyclonal antibody to assay for the amount of VLPs remaining in each well after detergent treatment. The Gag cleavage products capsid (CA) and matrix (MA) are labeled. For immature VLPs, the Gag-GFP construct is observed as two bands: (1) Gag-GFP (the parental fusion protein) and (2) full length Gag (arising from cleavage of Gag-GFP and consistent with previous observations using Gag fusion proteins) (Lindwasser and Resh, 2004). Experiment was performed twice with identical results.

only when treated with Tween 20 (Fig. 4, right panel). Treatment of either type of VLP with Empigen resulted in complete disruption of VLP membranes and the absence of detectable Gag protein. These results are consistent with the disruption profiles obtained by biosensor and confirm that mature VLPs show increased sensitivity to disruption by Triton X-100.

Remarkably, we also observed that even in ‘mature’ VLPs, a fraction of uncleaved Gag polypeptide was selectively retained following exposure to Triton X-100, while the mature Gag protein

subcomponents (Fig. 4, CA and MA) were washed away. The presence of uncleaved Gag in mature VLP preparations is not surprising, as both cleaved and uncleaved Gag proteins co-exist within a population of ‘mature’ VLPs, with up to 30% immature VLPs (Luftig and Yoshinaka, 1978; Yoshinaka et al., 1980). The selective retention of uncleaved Gag over cleaved Gag is consistent with the detergent resistance of immature VLPs, even when coexisting within the larger population of mature VLPs. Interestingly, both uncleaved and cleaved forms of Gag in the mature

VLPs were removed by Empigen detergent, consistent with the ability of Empigen to remove all VLPs from the biosensor surface (i.e. down to 0% signal in Fig. 2E). These results suggest that the residual amount of VLPs remaining on the biosensor surface after treatment of mature VLPs with Triton X-100 (i.e. down to 20–40% signal in Fig. 2A) is likely due to VLPs containing immature Gag within the larger population of mature VLPs. Maturation of the Gag core protein thus explains the sensitivity of VLP membranes to detergent in both assays and even within subpopulations of the same VLP preparation.

Discussion

Detergents are commonly employed in research and clinical practice for sanitizing surfaces and fluids against enveloped viruses. On the basis of their disruptive activity in other membrane types, it has largely been assumed that detergents act as lytic agents on the viral membrane. However, the ability of different detergents to rupture viral membranes has never before been directly investigated. In this study, we demonstrate that exposure to many detergents, including Triton X-100, BOG, CHAPS, NP-40, and Empigen, at or near their CMC, results in structural disruption of virion membranes. Remarkably, however, VLP membranes appeared highly resistant to complete lysis by a significant number of detergents, including Tween 20, Tween 80, Lubrol, and saponin. Only at very high concentrations of Tween 20 and with prolonged exposure was some gradual rupture observed, and even then it did not appear related to the CMC. Our results suggest that viral inactivation by detergents likely involves complete lysis in some cases, but for other detergents, likely involves other mechanisms such as localized pore formation.

Unlike pure lipid bilayers, retroviral membranes interact with, and are formed by, the structural proteins of the virus. In the case of retroviruses, these structural proteins (Gag) have been well-studied for their quaternary structure (Briggs et al., 2006a, 2006b; Yeager et al., 1998; Zanetti et al., 2006), structural stability (Park and Morrow, 1993; Stewart et al., 1990; Welker et al., 2000), and membrane interactions (Dalton et al., 2007; Ono et al., 2000; Zhou and Resh, 1996). Although it is known that isolated immature Gag cores exhibit greater structural stability compared to mature Gag cores (Kol et al., 2006, 2007; Stewart et al., 1990), there have been no previous reports that Gag can influence the stability or retention of the lipid bilayer surrounding the core. We were therefore surprised to find that VLPs containing immature (unprocessed) core proteins were significantly more resistant to detergent lysis than VLPs with mature cores. A possible mechanism for explaining how Gag processing influences detergent sensitivity is that immature Gag could be holding the entire virion structure together more tightly, serving as a more rigid internal scaffold to hold the lipid bilayer in place. This is supported by our observation that the point of first rupture is nearly identical for mature and immature VLPs, suggesting that lytic detergents initiate viral membrane rupture regardless of core structure but that the maturity of the core structure determines whether the lipid bilayer completely lyses and releases from the Gag core. Alternatively, a more rigid, immature Gag core could directly influence the fluidity of the lipid membrane. This notion is supported by atomic force microscopy studies showing that immature HIV-1 virions are significantly more 'stiff' than mature particles (Kol et al., 2007). Maturation of the HIV-1 Gag core has also been shown to affect the fusogenic ability of the Env glycoprotein. In particular, the Env complex was shown to be non-fusogenic in immature virions and becomes activated in mature particles due to cytoplasmic tail proteolysis (Murakami et al., 2004).

Taken together, these observations raise the intriguing possibility that Gag processing represents an important evolutionary adaptation for enveloped viruses which helps promote their survival, increasing the structural stability of nascent viral particles and restricting their infectivity until proper release from the host cell.

The loss of mass observed on the biosensor following treatment of VLPs with detergent is interpreted here as membrane rupture. Alternatively, the loss of mass could be interpreted as a weakening of the antibody–antigen interaction by the detergent leading to release of the VLPs from the biosensor surface. However, we believe this scenario is highly unlikely for several reasons. First, detergents such as Triton X-100 and Empigen are well known to disrupt lipid membranes and the abrupt loss of mass we observe occurs at or near the CMC of each detergent, suggesting that mass loss is specifically due to micellization of the VLP membranes. Second, we observe nearly identical mass losses for detergent-treated VLPs using two different capture antibodies against two very different membrane proteins (CD81 and CXCR4). Finally, the shear forces on captured VLPs in a laminar flow environment are expected to be small and to not contribute to particle detachment (Haun and Hammer, 2008).

Previous studies characterizing DRMs (lipid rafts) (Roper et al., 2000; Schuck et al., 2003) provide important insights regarding the detergent resistant properties of viral membranes. Both DRMs and viral membranes contain high quantities of cholesterol, sphingolipids, and saturated lipids that allow for the packing of lipids at high densities (a liquid-ordered phase) (Aloia et al., 1988, 1993; Brown and Rose, 1992; Munro, 2003; Pike et al., 2002; Polozov et al., 2008). A common characteristic of the detergents that fail to rupture VLP membranes in our studies is a structurally large polar head group, suggesting an inability of these detergents to effectively penetrate these tightly-packed lipid bilayers, similar to DRMs (de Foresta et al., 1989). It is important to note, however, that DRMs and retroviral membranes are structurally different. DRMs are often defined by their method of detergent extraction in the laboratory, so can possess different properties depending on the methods used for their extraction (Babychuk and Draeger, 2006; Chamberlain, 2004; Schuck et al., 2003). Retroviruses, by contrast, are self-assembled by Gag without laboratory manipulation, so they represent a model for isolating and studying naturally occurring lipid domains in the cell membrane.

The response of mature VLPs to detergents revealed that lysis did not usually progress to completion (i.e. intact mature VLPs did not decrease to 0% except in the case of Empigen treatment). This may be explained by the existence of a fraction of immature VLPs even in populations of 'mature' VLPs. Our observation that uncleaved Gag molecules are selectively retained during lysis of mature VLPs (Fig. 4) supports this interpretation.

Non-enveloped viruses, such as rhinoviruses and adenoviruses, maintain their structural stability during transmission in aerosols, biological fluids, and inhospitable environmental conditions (Dick et al., 1987; Jennings and Dick, 1987; Roden et al., 1997; Sene et al., 1995). In contrast, enveloped viruses are inherently more fragile due to their lipid bilayer so may have evolved mechanisms to maintain their stability during transmission (Lowen et al., 2007; Polozov et al., 2008). To the extent that the role of viral lipid membranes in viral stability and transmission can be understood, characterizing them may reveal new avenues for pharmaceutical intervention. Numerous antibiotics and lytic peptides act against the unique composition of bacterial membranes (Wimley and Hristova, 2011), yet few drugs have ever been developed specifically against viral membranes, despite the fact that they have unique biophysical properties required for virus infectivity and resilience. The recent characterization of two small molecule compounds that act directly on enveloped virus

membranes illustrates that viral membranes represent viable targets for drug development (St Vincent et al., 2010; Wolf et al., 2010). From this perspective, differences in the biophysical properties of structurally divergent enveloped viruses, such as flaviviruses, may be especially interesting to compare in future studies. The detection assays developed here may be useful tools for such studies, as well as for the study of other lytic agents interacting with enveloped viruses. Our results suggest that the development of microbicides and drugs that specifically target the unique properties of the virus membrane represents a feasible strategy for eliminating infective viral particles.

Materials and methods

Materials

The following detergents were obtained commercially: Triton X-100, Tween 20 (Fisher Scientific, Fair Lawn, NJ.), β -octylglucoside (BOG) (Sigma, St. Louis, MO), saponin, CHAPS, Lubrol (MP Biomedicals, Solon, OH), NP-40 (Pierce, Rockford, IL), and Empigen BB (Calbiochem, La Jolla, CA). The anti-CD81 (TAPA-1) antibody, JS-81, was purchased from BD Biosciences (San Diego, CA) and the 12G5 antibody, specific for the chemokine receptor CXCR4, was obtained from Dr. James Hoxie at the University of Pennsylvania. Anti-Gag polyclonal antibody was derived by immunization of chickens with SDS-PAGE purified MLV Gag (p70) (Cocalico Biologicals, Inc., Reamstown, PA).

VLP production, purification, and characterization

Virus-like particles (VLPs) were produced using a plasmid (pCGP) encoding the structural (gag) and non-structural (pol) genes of MLV (Soneoka et al., 1995). Immature VLPs were generated using a modified pCGP plasmid in which epitope or fluorescent tags were cloned as an in-frame fusion to the gag ORF to eliminate the pol gene. To accomplish this, an NheI restriction site was introduced after the gag gene using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). pCGP plasmids were used to transfect HEK-293T cells, and supernatants containing VLPs were harvested and passed through a 0.45 μ m filter as previously described (Balliet and Bates, 1998; Hoffman et al., 2000; Rucker, 2003). Filtered supernatants were purified through 20% sucrose cushions and pellets were re-suspended in 10 mM HEPES pH 7.5 and stored at 4 °C. VLPs expressing CXCR4 (commercially referred to as “Lipoparticles”) on their surface were produced using similar methods, but by co-transfecting a CXCR4 expression plasmid. Each lot of VLPs produced was assayed for homogeneity by dynamic light scattering (DLS) using a Protection DynaPro DLS.

Biosensor stability studies

All biosensor surface attachments were performed in PBS buffer alone or supplemented with 1 mg/ml BSA (running buffer) at 25 °C using a Biacore 2000 optical biosensor (GE Healthcare, Piscataway, NJ). VLPs were captured using monoclonal mouse antibodies, either JS-81 (against CD81) or 12G5 (against CXCR4), which were captured using a goat anti-mouse GAM Fc polyclonal antibody (Jackson ImmunoResearch Westgrove, PA) immobilized on the chip surface as previously described (Willis et al., 2008). Briefly, GAM Fc was diluted 1:10 in 0.1 M sodium acetate, pH 4.0 and attached to a Biacore C1 chip following a 10 min activation of surface carboxyl groups using a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1 M) and *N*-hydroxysuccinimide (NHS) (0.25 M) at 10 μ l/min.

Following attachment, the remaining surface carboxyl groups were quenched with 80 μ l of 1 M ethanolamine, pH 8.5, at 10 μ l/min. The capture antibody used was diluted to 5 μ g/ml in running buffer. About 20 μ l of capture antibody was injected at 10 μ l/min, followed by 5 μ l of VLPs at 1 μ l/min. Detergent solutions were made in running buffer and were introduced to the system in six 5- μ l injections of increasing concentration at 20 μ l/min. Detergents were tested for 15 s (unless otherwise indicated), with a 2-min wash between each injection. VLPs and capture antibodies were removed following each stability experiment using two 25- μ l pulses of 1% Triton X-100 in PBS followed by three 15- μ l pulses of 100 mM H_3PO_4 at 50 μ l/min. Data analysis was performed using BiaEvaluation 3.0 (GE Healthcare, Piscataway, NJ).

Western blot detection of disrupted VLPs

Mature and immature VLPs were captured onto 96-well ELISA plates overnight at 4 °C using 10 μ g/ml WGA lectin (Sigma, St Louis, MO). VLPs were washed 3 $\times$ with PBS and treated with 1% Empigen, 1% Tween 20, or 1% Triton X-100 for 10 min at room temperature with shaking. Following three PBS washes, the contents of each well were lysed in 5 $\times$ sample buffer (2 M Tris HCl pH 6.8, 10% SDS, 20% glycerol, 0.005% bromophenol blue, and 50 mM DTT), boiled for 5 min, and loaded onto a 4–20% SDS-PAGE gradient gel (NuSep, Bogart, GA). SDS-PAGE gels were run at 100 V for 1.5 h followed by transfer to PVDF membranes at 45 V for 1.5 h at room temperature. Membranes were probed with a polyclonal anti-Gag antibody (1:1000) followed by three washes with PBS-T (PBS+0.05% Tween). Blots were then incubated with an HRP-conjugated secondary antibody, washed 3 $\times$ with PBS-T, followed by the addition of Femto HRP substrate (Pierce, Rockford IL). Chemiluminescent detection was performed using a Fluorchem imager (AlphaInnotech, now Protein Simple, Santa Clara, CA).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2012.08.023>.

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