Appraising the apoptotic mimicry model and the role of phospholipids for poxvirus entry

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Entry of vaccinia virus (VACV) into cells occurs by fusion with the plasma membrane and via a low pH-dependent endosomal pathway, presumably involving unidentified cellular receptors. In addition to ≈25 viral proteins, the membrane of VACV mature virions contains several phospholipids including phosphatidylserine (PS). A recent model posits that PS flags virions as apoptotic debris to activate a common cellular uptake pathway to gain cell entry, perhaps through an interaction with a PS-specific cell surface receptor. To evaluate the apoptotic mimicry model, we reconstituted the membrane of detergent-extracted virions with several different phospholipids. Although the ability of the L-stereoisomer of PS to reconstitute infectivity was confirmed, the nonbiologically relevant D-stereoisomer of PS, and phosphatidylglycerol, which are not normally present in the virion membrane, functioned as well. Regardless of which phospholipid reconstituted infectivity, virus entry was inhibited by a neutralizing monoclonal antibody to a virion surface protein and by the drugs blebbistatin and bafilomycin A1, suggesting that in each case virus uptake was specific and occurred by a similar mechanism involving macropinocytosis and a low-pH endocytic pathway. Lipid-reconstituted and nonreconstituted, membrane-extracted virions were equally capable of binding to cells. However, the physical association of phospholipids with virus particles during membrane reconstitution correlated directly with rescue of particle infectivity and cell entry capability. Our results support a role for PS in poxvirus entry, but demonstrate that other phospholipids, not known to signal uptake of apoptotic debris, can function similarly.

blebbing | endocytosis | macropinocytosis | phosphatidylserine | vaccinia virus

Poxviruses are large, complex DNA viruses that are of considerable interest from ecological, medical, and scientific points of view because of their wide distribution among vertebrate and invertebrate species, ability to cause disease, and replication entirely within the cytoplasm of infected cells (1). Vaccinia virus (VACV), used as the smallpox vaccine, is the prototypic member of the poxvirus family and encodes ≈200 proteins including many required for RNA and DNA synthesis. The major infectious form of VACV, known as the mature virion (MV), is comprised of a 195-kbp dsDNA genome and ≈80 proteins, including a complete transcription system. A lipid membrane, with ≈25 associated proteins, surrounds the core of the MV (2). During the transit through the cytoplasm, some MVs acquire two additional membranes, one of which is lost during exocytosis to form the enveloped virion (EV). An EV is essentially an MV with an additional membrane containing at least six unique proteins (3). MVs can be released directly by cell lysis, and both MVs and EVs are infectious. Whereas MVs can directly fuse with the cell membrane to release the core into the cytoplasm, the outer membrane of EVs must be disrupted first (4). In agreement with this model, the VACV-encoded proteins involved in cell entry/membrane fusion all are located in the MV membrane (5).

The majority of VACV entry studies have been carried out with MVs, because they are more abundant and stable than EVs, which mostly remain attached to the outer surface of the plasma membrane of the parental cell (6). MVs enter cells by a low pH endosomal pathway (7) and through the plasma membrane (8), depending to some extent on the VACV strain (9). Initial attachment of MVs to cells is mediated in part through viral membrane proteins, which bind glycosaminoglycans or laminin, but are individually nonessential (10–13). An additional 11 or more proteins comprise or are associated with a complex that mediates or activates the membrane fusion step of entry (14–23). Although at least 10 of these proteins are each essential for entry, their precise roles in the fusion process are not known; however, involvement of one protein in binding to the cell has been suggested (24).

In addition to proteins, the MV membrane contains several phospholipids, including phosphatidylserine (PS) (25, 26). In a remarkable and long neglected series of experiments, Ichihashi and Oie (27) and Oie (28) showed that the loss of MV infectivity on Nonidet P-40 detergent extraction of lipids could be partially rescued by incubation of the extracted virions with exogenous lipids, including crude mammalian cell membrane preparations, pools of purified cell phospholipids, or purified PS. These results implicated host cell-derived phospholipids, and specifically PS, in MV entry. PS, which is normally present in the inner leaflet of the plasma membrane, is translocated to the outer leaflet during apoptosis and recognized as an "eat me" signal for phagocytic uptake of apoptotic bodies (29). Mercer and Helenius (30) provided evidence for the uptake of VACV by macropinocytosis and concluded that PS specifically flags virions as apoptotic debris to specifically trigger this cell uptake mechanism.

In recent years, several candidate eat-me receptors (Tim1, Tim4, and stabilin2) have been described as highly specific for PS (31–34). For many reasons, including the ability to express reporter genes, VACV could serve as an excellent surrogate for an apoptotic body to further identify eat-me receptors. However, before proceeding, we wanted to determine the lipid specificity for VACV entry, because there is evidence that PS is recognized by macrophages in a stereospecific manner on apoptotic bodies (35). Surprisingly, we found that the nonbiologically relevant D stereoisomer of PS (PS-D) and even phosphatidyglycerol (PG) are capable of reconstituting MV infectivity, as is the L stereoisomer of PS (PS-L). Our results confirmed the importance of phospholipids in VACV entry, but demonstrated that the putative "receptor recognition" of VACV phospholipids is not highly structure-specific. The relevance of these findings to uptake of apoptotic debris by nonprofessional phagocytic cells in general needs to be determined.

Results

Reconstitution of Detergent-Extracted Virions. The recombinant VACV used for these experiments, WRvFire, encodes firefly luciferase (LUC) regulated by a strong early/late promoter, which provides a rapid and sensitive method of detecting virus entry into cells (7). Following previous protocols (28, 30), purified MVs were extracted with Nonidet P-40 detergent and

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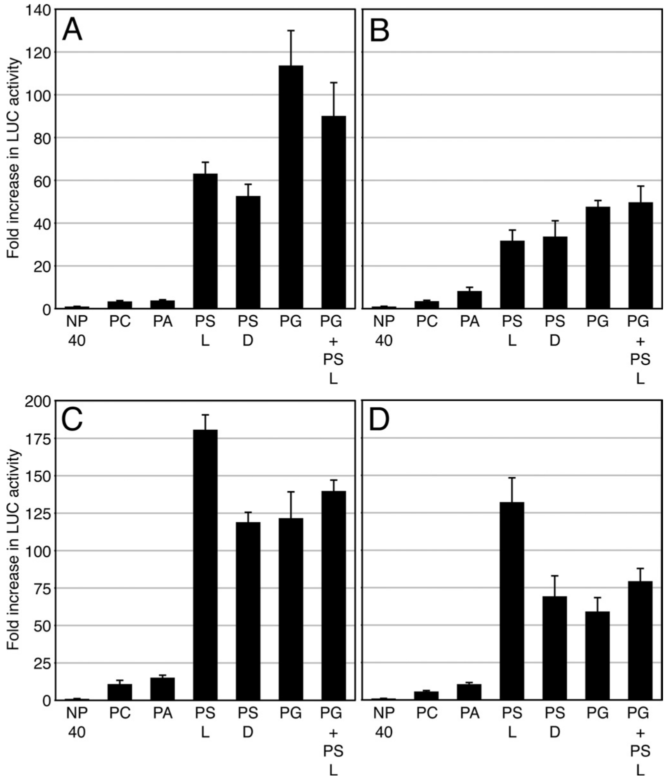

Fig. 1. Cell entry of Nonidet P-40-treated and lipid-reconstituted VACV. Purified MVs encoding LUC were extracted with Nonidet P-40 detergent and either unreconstituted (Nonidet P-40) or reconstituted with PC alone or a 1:1 mixture of PC and the indicated phospholipid (PA, PS-L, PS-D, and PG) or the combination of PG + PS-L. Virus was adsorbed to BS-C-1 (*A* and *B*) or HeLa (*C* and *D*) cells at neutral pH at 4 °C and then incubated with pH 7.4 (*A* and *C*) or pH 5.0 (*B* and *D*) buffer at 37 °C for 3 min. Incubations were then continued for 2 h at neutral pH, and virus entry was determined by measuring LUC activity. The ordinate shows ratios of LUC activity induced by lipid-reconstituted versus nonreconstituted (Nonidet P-40) virions for each pH condition as indicated. The presented results are representative of several independent experiments.

then reconstituted with lipids. Entry was determined by measuring LUC activity after adsorbing the reconstituted MVs at 4 °C to BS-C-1 and HeLa cells, and then incubating virus-bound cells at 37 °C for 2 h. PC alone was ineffective at promoting entry of Nonidet P-40-treated virions (Fig. 1 *A* and *C*), but it was used in a 1:1 ratio with a second phospholipid to enhance dispersion of the latter. For simplicity, we only refer to the second phospholipid in the text and figures. Although we confirmed the ability of PS-L to reconstitute virions and allow virus entry, surprisingly, the nonbiologically relevant PS-D and PG were also effective, and the combination of PS-L and PG did not enhance entry above the levels observed for PG alone (Fig. 1 *A* and *C*). Phosphatidic acid (PA), like PC, was ineffective at promoting entry of Nonidet P-40-treated virions (Fig. 1 *A* and *C*). The findings with the entry assay were reproduced by extending the incubation and measuring virus infectivity in a plaque formation assay. Restoration of infectivity was 5–10% with PS-L, PS-D, or PG, similar to levels obtained by Ichihashi and Oie (27) and Oie (28). The inability to regain full infectivity may be caused by partial loss of viral proteins by the Nonidet P-40 extraction (36).

The results described so far were carried out under neutral pH conditions, in which MVs of the Western Reserve (WR) strain mainly enter by a low pH-dependent endocytic pathway (7). After adsorption and binding of VACV to the cell surface, entry can be greatly accelerated by brief treatment with pH 5.0 buffer, which promotes the direct fusion of virions with the plasma membrane (7). However, entry of lipid-reconstituted virions was not enhanced by brief low pH buffer after virus binding to either BS-C-1 or HeLa cells (Fig. 1 *B* and *D*). Absence of low pH stimulation at the cell surface was previously found for VACV WR that had been activated by pretreatment with either low pH or proteases (37) and for other strains of VACV (9).

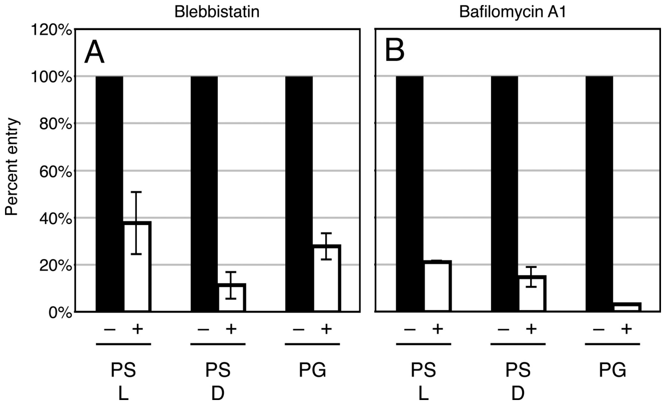

Fig. 2. Cell entry of lipid-reconstituted VACV depends on macropinocytosis and involves the low-pH endocytic pathway. Cells were pretreated in the absence (−) or presence (+) of 75 μM blebbistatin (*A*) or 50 nM bafilomycin A1 (*B*) before virus adsorption. Nonidet P-40 detergent-treated MVs were reconstituted with a 1:1 mixture of PC and the indicated phospholipids. Entry of lipid-reconstituted virions into cells was then determined as described in Fig. 1, except that the drug concentrations were maintained during virus adsorption and incubation. LUC values obtained in the absence of drug were set to 100%. The presented results are representative of several independent experiments.

Entry of Lipid-Reconstituted Virions Through the Low-pH Endosomal Pathway. Previous studies had shown that entry of VACV strain WR is inhibited by blebbistatin, which prevents macropinocytosis (30), and bafilomycin A1, which prevents acidification of endosomes (7). We found that, regardless of the phospholipid used to reconstitute infectivity, virus entry was significantly decreased by blebbistatin (Fig. 2*A*) and bafilomycin A1 (Fig. 2*B*). These results suggested that entry of the virions reconstituted with PS-D or PG, and PS-L, occurred by macropinocytosis and involved the low-pH endocytic pathway.

Entry of Lipid-Reconstituted Virions Is Inhibited by a Neutralizing mAb. Further experiments were carried out to determine whether the entry of lipid-reconstituted virions involved virus-specific mechanisms. The L1 protein, an integral transmembrane component of the MV membrane, is required for viral membrane fusion and cell entry (21) and antibodies that target L1 have neutralizing activity (38–40). The sensitivity to a L1 mAb of virions reconstituted with PS-L (Fig. 3*B*), PS-D (Fig. 3*C*), and PG (Fig. 3*D*) was similar to that of untreated virions (Fig. 3*A*).

Binding of Lipid-Reconstituted Virions to Cells. A flow cytometry-based assay (9) was used to determine whether lipid reconstitution increased the binding of virions to cells. Recombinant VACV MVs with YFP fused to the viral A4 core protein were extracted with Nonidet P-40 detergent, reconstituted with different phospholipids, and then allowed to bind to cells in the cold for 1 h. Cells were washed to remove unbound virions, and then analyzed by flow cytometry. Approximately 60–70% of the cells scored positive for yellow fluorescence regardless of whether the Nonidet P-40-extracted virions were reconstituted with lipids (Fig. 4*A*), in agreement with previous data (30).

Association of Phospholipids with Detergent-Extracted Virions. The acquisition of phospholipids by Nonidet P-40 detergent-extracted

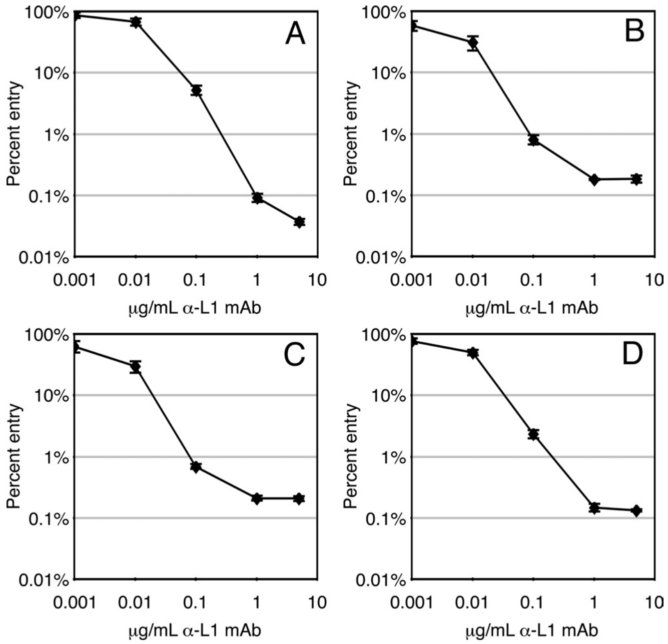

Fig. 3. Inhibition of cell entry of lipid-reconstituted VACV by a neutralizing mAb. Equivalent amounts of untreated control (*A*), PC + PS-L (*B*), PC + PS-D (*C*), and PC + PG (*D*) lipid-reconstituted virions were incubated with increasing concentrations of anti-L1 mAb as indicated on *x* axis. Virus–antibody complexes were then adsorbed to cell monolayers for 90 min at 37 °C, at which point cells were harvested to quantify LUC activity as a measure of entry. The data are represented as the percentage of the entry value obtained for each virus in the absence of anti-L1 protein antibody and plotted on the *y* axis.

virions was determined by analyzing their densities after sedimentation in cesium chloride gradients. Untreated, control virions remained near the top of the gradient with a density of 1.26 g/mL (Fig. 4*B*), corresponding to the known density of VACV MV particles (41). After Nonidet P-40 treatment of purified MVs, undetectable levels of phospholipids, neutral lipids, and cholesterol remain associated with the virus particles (28). Consequently, Nonidet P-40 detergent-treated virions sedimented to a lower position (1.34 g/mL) than MVs (Fig. 4*B*). Incubation with PC alone did not shift the sedimentation of Nonidet P-40 detergent-treated virions from their density of 1.34 g/mL (Fig. 4*B*), correlating with their lack of infectivity. In contrast, reconstitution with PS-L, PS-D, or PG each shifted the density toward that of untreated virions (Fig. 4*B*), demonstrating a physical association of these phospholipids with reconstituted VACV that correlated with acquisition of infectivity.

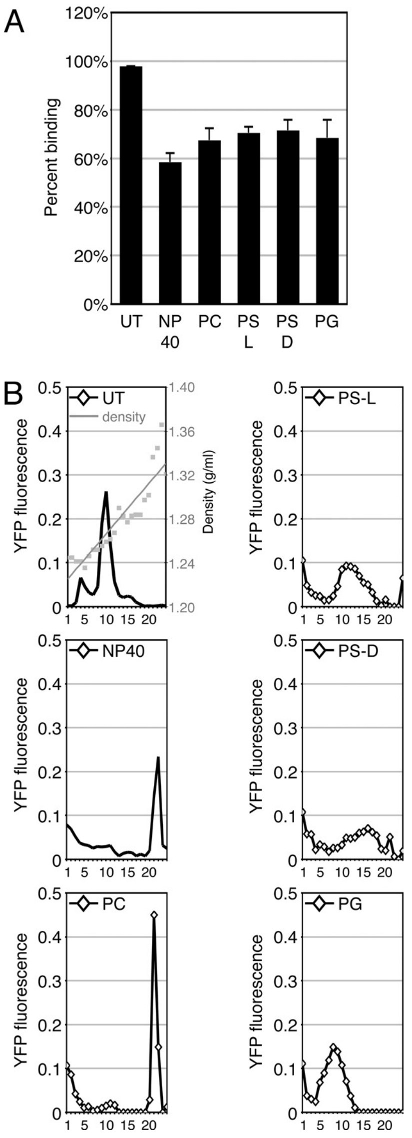

Fig. 4. Cell binding properties and lipid acquisition of Nonidet P-40-treated and lipid-reconstituted VACV. Nonidet P-40 detergent-extracted MVs were reconstituted with a 1:1 mixture of PC and the indicated phospholipids. (*A*) Equivalent amounts of YFP-labeled control and lipid-reconstituted virions were adsorbed to cell monolayers for 60 min at 4 °C and then processed for flow cytometry. The percentage of virus-bound cells (*y* axis) is shown for the individual virions tested on the *x* axis. Data are representative of at least three independent experiments. (*B*) Cesium chloride sedimentation gradient profiles of untreated (UT), membrane-extracted (Nonidet P-40), and lipid-reconstituted (PC, PS-L, PS-D, and PG) virions. Fractions were collected from the top, and fraction numbers are indicated on the *x* axis of each plot. Data are presented as the percentage of total virus (*y* axis) in each gradient fraction as determined by YFP flourescence. The densities (g/mL, right *y* axis) of individual gradient fractions are shown as gray squares and a gray trend line in the UT panel.

Discussion

Viruses rely heavily on their host cells for replication and consequently can provide important insights into cellular processes. Indeed, much of what we know regarding membrane fusion has come from studies with viruses (42). Similarly, viruses are providing a wealth of information on mechanisms of endocytosis (43). Depending on size and other factors, viruses use distinct endocytic pathways. VACV is one of several viruses thought to use macropinocytosis, an actin-dependent process that leads to internalization of extracellular fluid and particles (7, 30, 44, 45). Mercer and Helenius (30) proposed that PS on the surface of VACV virions mimics a defining feature of apoptotic bodies and initiates a signaling cascade resulting in plasma membrane blebbing and macropinocytosis. Thus, VACV might serve as a useful surrogate to understand how apoptotic bodies are recognized and internalized. To potentially exploit this system, we used a recombinant VACV that rapidly expresses LUC after entry of the virus core into the cytoplasm (7).

The specificity of PS for VACV entry was suggested by Oie (28), who found that other lipids, namely PC, phosphatidylethanolamine, phosphatidylinositol, lysolecithin, sphingomyelin, and acyl bis(monoacylglycero)phosphate were unable to reconstitute the infectivity of Nonidet P-40-extracted virions. Although we were able to confirm the ability of PS to reconstitute entry and infectivity, we were surprised to find that the nonbiologically relevant PS-D worked as well as PS-L. Also, PG worked even better than PS-L in most experiments, whereas PC alone and PA were ineffective. Regardless of whether PS-L, PS-D, or PG was used for reconstitution, entry was inhibited by blebbistatin and bafilomycin A1, suggesting that macropinocytosis and low pH-dependent endocytosis were involved. Also, the restoration of infectivity correlated with the ability of phospholipids to associate with virions, raising the question of whether the phospholipids need have an additional role. The lipids did not mask the membrane proteins as entry of reconstituted virions was inhibited by a VACV-neutralizing mAb.

Choline-containing lipids such as PC and sphingomyelin are enriched in the external leaflet of the plasma membrane and the corresponding luminal side of intracellular organelles, whereas PS and phosphatidylethanolamine, and several more minor phospholipids, are enriched to varying extents on the cytoplasmic side of membranes (46). This phospholipid asymmetry of the plasma membrane is lost during an early stage of apoptosis (47). Annexin V recognizes certain phospholipids, including PS and phosphatidylethanolamine, and for this reason, is extensively used as a marker of apoptosis (48). However, the presence of PS on the surface of VACV, as suggested by binding of annexin V (30), is likely to be a consequence of the intracellular site of viral membrane formation (2), rather than of apoptosis.

Our finding that PS-L is not specifically required for entry of reconstituted virions contrasts with studies on uptake of apoptotic bodies by macrophages (35) and the ligand specificity of some recently identified PS-specific receptors (31–34). These PS receptors are unlikely to be involved in VACV entry based on their tissue distribution and ligand specificity. For example, Tim1 and Tim4 both bind PS-L exclusively and have no affinity for PC, PA, or PG (34). Stabilin2 binds and mediates uptake of apoptotic bodies in a PS-L-specific manner as targets coated with PC, PA, or PG are not engulfed by stabilin2-expressing cells (32). Also, no significant uptake of authentic apoptotic targets bearing PS-D was observed on presentation to phagocytic macrophages (35). Thus, a putative phospholipid receptor involved in VACV entry would not exhibit stringent specificity for phospholipid structure.

Materials and Methods

Cells and Viruses. African green monkey kidney BS-C-1 and HeLa cells were maintained in Eagle's minimal essential medium (EMEM) (Quality Biological) supplemented with 2.5% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (EMEM + 2.5% FBS). All experiments were performed with the VACV strain WR (ATCC VR-1354). The recombinant VACV encoding firefly LUC under a synthetic early/late promoter (WRvFire) and the recombinant VACV expressing YFP fused to the A4 core protein (WR A4-YFP) have been described (7, 49).

Virus Purification. BS-C-1 cells were infected with VACV WRvFire or WR A4-YFP, and at 48–72 h postinfection, MVs were isolated (50). Briefly, infected cells were subjected to Dounce homogenization and MVs were purified by sedimentation through two 36% (wt/vol) sucrose cushions followed by one sedimentation on a 25–40% (wt/vol) continuous sucrose gradient; the visible virus band was collected, and virus was pelleted and stored at −80 °C. On thawing for experiments, virus was sonicated on ice for 1 min before use.

Liposome Generation. Phospholipids were purchased from Avanti Polar Lipids. Lipids in chloroform were mixed at 1:1 molar ratio of PC and a second phospholipid unless otherwise specified. Lipids were dried under nitrogen gas, resuspended in PBS, sonicated briefly, and stored at 4 °C for future use.

Lipid Reconstitution of VACV MV. Similar to the methods of Oie (28) and Mercer and Helenius (30), purified MVs (1×10^9 plaque-forming units) were extracted with 0.5% Nonidet P-40 detergent (Sigma–Aldrich) in 100 mM Tris, pH 9 and 0.05% BSA for 1 h at 37 °C. Virions were subjected to sedimentation ($16{,}000 \times g$, 17 °C, 30 min), and the virus pellet was washed twice with PBS plus 0.05% BSA. Aliquots of Nonidet P-40 treated virus that corresponded to 1×10^6 plaque-forming units were resuspended in PBS plus 0.05% BSA and incubated with 200 μg of liposomes for 2 h at 37 °C. Virus was purified by sedimentation through 36% (wt/vol) sucrose ($16{,}000 \times g$, 4 °C, 60 min), and virus pellets were resuspended in PBS plus 0.05% BSA for subsequent assays.

LUC-Based Entry Assay. BS-C-1 (1.3×10^5 per well) and HeLa (2.0×10^5 per well) cells in 24-well plates were chilled to 4 °C before virus adsorption. Equivalent amounts of control and lipid reconstituted WRvFire viruses were adsorbed in cold EMEM without serum for 1 h at 4 °C. Cells were washed with cold PBS to remove unbound virions and incubated with prewarmed EMEM + 2.5% FBS for 2 h at 37 °C. Cells were washed with PBS and then incubated with Cell Culture Lysis Reagent (Promega) for 30 min at room temperature with gentle agitation. LUC activity in cellular extracts was measured according to the manufacturer's protocol (Promega) and quantified on a Berthold Sirius luminometer (Berthold Detection Systems).

Stimulation of Virus Entry by Low-pH Treatment. Stimulation of virus entry by low-pH treatment was performed as described (7). Briefly, after washing to remove unbound virions as described above for the LUC entry assay, cells were incubated for 3 min at room temperature in either PBS with Ca^{2+} and Mg^{2+} at pH 7.4 or PBS with Ca^{2+} and Mg^{2+} supplemented with 1 mM 2-morpholinoethane-sulfonic acid adjusted to pH 5 with HCl. After removal of buffers, the pH was neutralized by one wash with EMEM + 2.5% FBS. Cells were then incubated in prewarmed EMEM + 2.5% FBS for 2 h at 37 °C. Cells were then prepared for the LUC entry assay as described above.

Bafilomycin A1 and Blebbistatin Treatment. HeLa cells seeded in 24-well plates were left untreated or pretreated with either 50 nM bafilomycin A1 (Sigma–Aldrich) or 75 μM blebbistatin (Sigma–Aldrich) for 30 min at 37 °C. Reconstituted WRvFire virus was adsorbed to cells at 4 °C and incubated at 37 °C as described above while maintaining the drug.

Anti-L1 mAb Neutralization Assay. Equivalent amounts of control and lipid reconstituted WRvFire viruses were incubated with increasing amounts of anti-L1 mAb (38) in EMEM without serum for 30 min at room temperature. Virus–antibody complexes were adsorbed to HeLa cell monolayers in 24-well plates for 90 min at 37 °C. Cells were harvested as described above to quantify LUC activity.

Flow Cytometry-Based Assay for Virus–Cell Binding. Equivalent amounts of control and lipid reconstituted WR A4-YFP viruses were incubated with HeLa cells in 24-well plates at neutral pH for 1 h at 4 °C. Cells were washed twice with cold PBS to remove unbound virus. Cells were harvested, fixed in 2% paraformaldehyde, and analyzed with a FACSCalibur flow cytometer using CellQuest (BD Biosciences) and FlowJo Software (Tree Star).

Sedimentation of Virus in Cesium Chloride Gradients. Control and lipid reconstituted WR A4-YFP virions were individually overlaid onto continuous cesium chloride gradients [≈1.29–1.22 g/mL (wt/vol)]. Gradients were subjected to sedimentation at $175{,}000 \times g$ for 4 h at room temperature in a SW41 Ti rotor (Beckman Coulter). Twenty-four fractions were collected from the top of the gradient. Individual gradient fractions were scanned for fluorescence [488 nm (excitation) and 530 nm (emission)] with a SpectraMax M5 fluorescence plate reader (Molecular Devices) to determine the presence of WR A4-YFP virions. Gradient fraction densities were determined with a refractometer. To validate virus detection by this method, gradient fractions were precipitated with trichloroethanoic acid and proteins therein resolved by SDS/PAGE for Western blot analysis with an antibody specific for a viral core protein.

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