HIV virions sense plasma membrane heterogeneity for cell entry

Sung-Tae Yang,1,2 Alex J. B. Kreutzberger,1,2 Volker Kiessling,1,2 Barbie K. Ganser-Pornillos,1,2 Judith M. White,1,3 Lukas K. Tamm1,2*

It has been proposed that cholesterol in host cell membranes plays a pivotal role for cell entry of HIV. However, it remains largely unknown why virions prefer cholesterol-rich heterogeneous membranes to uniformly fluid membranes for membrane fusion. Using giant plasma membrane vesicles containing cholesterol-rich ordered and cholesterol-poor fluid lipid domains, we demonstrate that the HIV receptor CD4 is substantially sequestered into ordered domains, whereas the co-receptor CCR5 localizes preferentially at ordered/disordered domain boundaries. We also show that HIV does not fuse from within ordered regions of the plasma membrane but rather at their boundaries. Ordered/disordered lipid domain coexistence is not required for HIV attachment but is a prerequisite for successful fusion. We propose that HIV virions sense and exploit membrane discontinuities to gain entry into cells. This study provides surprising answers to the long-standing question about the roles of cholesterol and ordered lipid domains in cell entry of HIV and perhaps other enveloped viruses.

INTRODUCTION

Cell membranes consist of numerous proteins and lipids exhibiting complex behavior that includes organization into dynamic nanodomains enriched in sphingolipids and cholesterol that are sometimes referred to as "lipid rafts" (1, 2). Accumulating evidence indicates that membrane domain organization plays a vital role in cell signaling and adaptive immune responses to combat infections by many pathogens (3–5). For example, HIV has evolved to exploit organized membrane domains to gain entry into host cells (6–9). It is well established that recognition and attachment of HIV to host cells are mediated by the binding of the viral envelope glycoprotein gp120 to the cell-surface receptor CD4 and a co-receptor CCR5 or CXCR4 (10, 11). After attachment, membrane fusion mediated by subunit gp41 of the envelope glycoprotein leads to cell entry. Because CD4 has been found to associate with detergent-resistant fractions of the cell membrane, it has been assumed that cholesterol- and sphingomyelin-rich rafts are platforms for HIV entry (12, 13). However, because lipids in cholesterol-rich regions are more tightly packed and more ordered than those in the surrounding membrane, these sites may seem energetically unfavorable for membrane fusion, thereby raising doubts about the involvement of ordered lipid regions in the fusion step of HIV entry.

Because ordered lipid domains are thought to be dynamically fluctuating nanoscopic assemblies of lipids and resident proteins in living cells, visualization of the potential involvement of these membrane regions upon entry of viral particles into intact cells remains technologically challenging (14, 15). In a first step toward solving this problem, we showed recently that model membranes with coexisting microscopic ordered and fluid lipid domains were useful in proving that the fusion peptide of gp41 interacts preferentially with ordered/disordered lipid domain boundaries and that these boundaries are also the preferred sites for fusion peptide–mediated membrane fusion (16). However, these discoveries raised the important question of whether the results obtained in this highly artificial model system could be translated to biological membranes that contain the HIV receptor and co-receptor and to virus particles bearing the full, trimeric, gp120/gp41 complex, that is, whether membrane domain boundaries would still be the preferred sites for virus attachment and/or membrane fusion at the plasma membranes of appropriate target cells. Therefore, we developed a new approach to measuring binding and fusion of HIV particles with plasma membranes to assess the role of membrane heterogeneity in these processes.

We used giant plasma membrane vesicles (GPMVs) derived from HeLa cells that stably express the CD4 receptor and CCR5 co-receptor (CD4+/CCR5+), investigated the lateral partitioning of both receptors in these membranes, and imaged the preferred regions of HIV binding and fusion at the single-particle level (Fig. 1A). GPMVs show temperature-dependent, micrometer-scale liquid-ordered/liquid-disordered (Lo/Ld) phase separation and have been extremely useful to study the dynamics and lateral distribution of resident membrane proteins (17, 18). Using this approach, we found that lipid order discontinuities in heterogeneous receptor- and co-receptor–containing plasma membranes are important for gp120-mediated receptor/co-receptor targeting and gp41-mediated membrane fusion.

RESULTS

Lateral distribution of CD4 and CCR5

A number of studies have been conducted to localize CD4 and CCR5 in lymphocyte cell membranes. Although the association of CD4 with lipid rafts is generally accepted, divergent conclusions have been reached regarding the raft localization of CCR5 (6, 12). Confirming previous results, we observed by immunofluorescence that CD4 co-localized with raft-resident ganglioside GM1 in CD4+/CCR5+ HeLa cells, whereas CCR5 colocalized only partially with GM1 (fig. S1). When we prepared GPMVs from CD4+/CCR5+ HeLa cells by gentle formaldehyde and dithiothreitol (DTT) treatment (movie S1 and fig. S2, A and B), which forms membrane blebs by releasing otherwise intact cell membranes from the cytoskeleton (17, 18), we found that most GPMVs showed microscopic Lo/Ld phase separation below 25°C (movie S2 and fig. S2, C to E). As expected from immunostaining of intact cells, CD4 and GM1 were substantially excluded from the Ld region in GPMVs, indicating the partitioning of CD4 into the Lo region.

1Center for Membrane and Cell Physiology, University of Virginia, Charlottesville, VA 22908, USA. 2Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA 22908, USA. 3Department of Cell Biology, University of Virginia, Charlottesville, VA 22908, USA.

*Corresponding author. Email: lkt2e@virginia.edu

Preferential localization of CCR5 at the boundaries between Lo and Ld phases occurred and was evident in cell-attached blebs and cell-detached GPMVs (Fig. 1, B and C). This result might explain the debate about the controversial association of CCR5 with lipid rafts: CCR5 is not associated with Lo or with Ld lipid phases but accumulates at the boundaries between them. Very similar results were obtained with an alternative procedure to induce membrane blebs by the application of small concentrations of \(N\)-ethylmaleimide (NEM) (fig. S3), suggesting that the results are independent of the blebbing agent and that the resulting GPMVs do not have serious membrane defects.

**Recognition of Lo/Ld membrane boundaries by HIV**

Next, we examined whether and how HIV envelope (Env) particles interact with GPMVs. Murine leukemia viruses (MLVs) pseudo-typed with HIV gp120/gp41 and fluorescently labeled with mKO-Gag were incubated with CD4\(^+\)/CCR5\(^+\) GPMVs. We visualized bound particles by epifluorescence microscopy and found that they migrate along...
the GPMV surfaces (Fig. 1D and movie S3). Staining the GPMVs with the Ld marker 3,3’-dilinoleoylcarbocyanine perchlorate (DiO) showed that most bound particles localize to Lo/Ld boundary regions in cell-attached (Fig. 1E, movie S4, and fig. S4) and cell-detached GPMVs (Fig. 1F and movie S5). We quantified the viorn bound to three different regions (Lo, Ld, and Lo/Ld boundaries), as shown in Fig. 1G. To assess whether the targeting of the viorn to the Lo/Ld interface is specific to HIV Env, we also performed similar experiments with MLVs pseudotyped with the Envelope G protein (glycoprotein) from vesicular stomatitis virus (VSV-G). VSV-G viorn preferentially bound to the pseudotyped HIV Env receptors (Fig. 2A). Demonstrating the specificity of the HIV/GPMV fusion system, we found that lipid mixing is strongly suppressed by the well-known HIV entry inhibitors maraviroc and enfuvirtide (Fig. 2B). Maraviroc inhibits binding to the co-receptor CCR5 (19), and enfuvirtide inhibits fusion by blocking the required conformational change of gp41 (20). Maraviroc reduced the targeting of HIV viorn to phase boundaries (Fig. 2C), further supporting the notion that CCR5 recognition occurs at these boundaries. By contrast, enfuvirtide had little effect on the boundary preference of HIV binding while still blocking fusion (Fig. 2D), suggesting that binding boundary alone is not sufficient for fusion; it requires the energy from refolding gp41 into the six-helix bundle structure as well.

We also observed fusion with GPMVs at the single-particle level. The fluorescence of many HIV Env viorn that were bound to Lo/Ld phase boundaries spread over time, indicating that the particles fused with the GPMVs (Fig. 2E). In addition, we carried out electron cryo-microscopy (cryoEM) in an attempt to directly visualize the process of fusion of viral particles with GPMVs. As previously observed for bare MLVs containing only Gag and Gag-Pol (21), the spherical or tennis racket–shaped particles showed a characteristic morphology with a cupped shape by the viral envelope (Fig. 2F). Representative electron micrographs obtained from four different incubations with two separate viral and two separate GPMV preparations show HIV Env particles in intimate contact with the GPMV surfaces (Fig. 2G and fig. S6). We also observed membrane perturbations in very confined areas of the GPMV lipid bilayer that may represent fusion intermediates (Fig. 2G). To ensure that the observed contacts were specific, controls with GPMVs lacking CD4 and CCR5 were mixed with HIV Env particles. Fifteen to 30 randomly selected GPMVs per grid were imaged for three different samples, and no virus-GPMV fusion events were observed. However, the present resolution of the cryoEM images does not permit a distinction between Lo and Ld phases and is thus not sufficient to determine whether fusion occurs at the Lo/Ld phase boundaries.

**Requirement of Lo/Ld phase coexistence for fusion**

Next, we examined whether the Lo/Ld phase coexistence on GPMVs is required for HIV Env particle binding and/or fusion. To disrupt Lo phase domains, we treated GPMVs with methyl-β-cyclodextrin (MβCD), which depletes cholesterol from the membranes. MβCD treatment of GPMVs led to a marked change from approximately circular Lo domains to corrugated shapes that are characteristic for bilayers in the gel phase (Fig. 3A and movie S6). However, cholesterol extraction did not significantly alter the overall surface expression levels of CD4 or CCR5 on GPMVs (Fig. 3B). Virion binding was quantified by directly counting the number of particles attached to GPMVs (Fig. 3C) or by a centrifugation-based assay (Fig. S7). Regardless of the method, particle binding was high and not diminished by cholesterol depletion of the GPMVs, indicating that the presence of Lo domains is not required for the binding of viorn. However, HIV Env viorn did not bind to GPMVs lacking CD4 and CCR5, further confirming that their attachment is mediated by these receptors rather than lipid phase separation.

Contrary to virion attachment, disruption of the Lo phase domains in GPMVs by MβCD significantly decreased the efficiency of fusion of HIV Env viorn with GPMVs (Fig. 3D). This result agrees with a previous report demonstrating a decrease of HIV cell entry after depletion of cholesterol from the host cell membrane (22). The inhibitory effect on fusion by the disruption of Lo phases and Lo/Ld phase boundaries in GPMVs suggests that the unique properties of these boundaries are responsible for attracting receptors and co-receptors and the high fusogenicity of HIV Env viorn at these sites. Thus, the same physical principles that promote fusion peptide–mediated fusion in lipid model membranes, namely, line tension and lipid packing defects at Lo/Ld phase boundaries (23), also provide a significant driving force for membrane fusion of HIV Env viral particles with biological membranes.

**Fusion inhibition by lipid perturbation**

Lysolipids, which adopt an inverted-cone molecular shape, have been reported to inhibit fusion in a wide variety of systems owing to their propensity to induce positive intrinsic membrane curvature (24, 25). Here, we tested whether lysolipids affect the lipid phase behavior of GPMVs and the ability of HIV to fuse with them. Lysosphingolysylinyl (lysoSM) partially dissolved the Lo domains of GPMVs into much smaller domains, whereas lysosphatidylcholine (lysoPC) had little effect on Lo domains (Fig. 4A and movie S7). Although both lysoSM and lysoPC share a choline headgroup and a similar molecular shape, they may differently affect line tension at phase boundaries. LysoSM is a lineactant that appears to reduce line tension, whereas lysoPC probably partitions more uniformly into the Ld phase with a lesser effect on line tension. HIV Env virion binding was not affected by lysoSM or lysoPC (Fig. 4B). However, lysoSM significantly reduced the fusion efficiency of HIV particles, whereas lysoPC had only a moderate effect (Fig. 4B). Together, these results again support the notion that the site of fusion is the domain boundary region where inhibitory lysoSM is enriched, but lysoPC is not enriched. The same effect could be triggered enzymatically. Treatment of GPMVs with phospholipase A2 (PLA2), which hydrolyzes glycerophospholipids, including PC to lysoPC, had little effect on the lipid phase behavior of GPMVs and HIV fusion, whereas sphingolipid ceramide N-deacylase (SCDase), which converts SM into lysoSM, dispersed the Lo domains, which eventually spread over the entire
GPMV surface and significantly inhibited fusion (Fig. 4, C and D). Note that the fluorescent lipid probe DiI always marks the Ld phases in these images. The apparent reversal of contrast in some images is due to different levels of cholesterol that can change the connectivity of Lo phases, as previously demonstrated in model systems (26).

To further probe the influence of lysolipids on lipid phases and HIV fusion in well-defined model membranes, we used giant unilamellar vesicles (GUVs) and large unilamellar vesicles (LUVs) composed of bSM/bPC/bPS/cholesterol (2:1:1:1) as models (16). Only lysoSM significantly changed the Lo domains in GUVs (Fig. 4E) and inhibited the fusion of LUVs with HIV fusion peptide–decorated virosomes, whereas lysoPC had only a small effect at the highest concentration (Fig. 4F). The domain edges first undulated before larger invaginations formed, eventually dispersing the domains into much smaller ones (movie S8). Regulating the size of lipid rafts by using lysosphingolipids has significant consequences not only on HIV entry but also on cell signaling. For example, it is well known that sphingosine-1-phosphate is a potent mediator of numerous signaling pathways (27) and the finding that these signaling mechanisms could be potentiated by raft regulation may be an interesting topic for future investigations. As previously reported, the recruitment of some proteins such as Ras and Rac-1 to domain boundaries may be more general and may contribute to the regulation of signaling pathways by regulating the nature and size of lipid rafts in these systems (28, 29).

**HIV binding and fusion to SPPMs**

Supported lipid bilayers generated from synthetic and natural lipids have been widely applied since their original introduction (30), including more recently to study fusion of single particles (16, 31). The planar geometry of supported membranes has the advantage over GUVs that a large extended membrane can be observed by total internal reflection fluorescence (TIRF) microscopy, and, therefore, that large numbers of single events can be simultaneously recorded with high signal-to-noise ratio in each experiment, leading to greatly improved statistics (32). To exploit this advantage for biological membranes as well, we have developed supported planar plasma membranes (SPPMs) for single-particle fusion studies (Fig. 5A). Our strategy for preparing SPPMs is to fuse GPMVs onto polymer-supported lipid monolayers that have been transferred from a Langmuir trough (33), which is different from previous methods (34) and preferentially orients membrane receptors with their binding sites accessible from solution. Similar to plain supported lipid bilayers (26, 35), SPPMs prepared in this fashion display micrometer-sized domains, in which raft and nonraft lipid components are laterally mobile (fig. S8). In agreement with the observations with GPMVs, the overlay of CD4 and CCR5 images in SPPMs shows that CD4 was substantially associated with Lo domains, whereas CCR5 was preferentially located at the edges of the domains (Fig. 5B and fig. S9). We observed by TIRF microscopy that single HIV Env particles bound predominantly to domain boundaries, whereas most VSV-G particles...
bound to Ld phases on SPPMs (Fig. 5, C and D). In addition, these experiments recapitulate the observation made with GPMVs that the location of HIV binding to SPPMs is determined primarily by the location of the receptor and co-receptor in these membranes and not by the lipid phases (Fig. 5E).

We observed large numbers of single-membrane fusion events of HIV Env particles with SPPMs (Fig. 5F and movie S9) and classified them as docking, hemifusion, or full fusion (Fig. 5G) in Lo, Ld, and Lo/Ld compartments of the SPPM. We observed no direct full fusion and very little hemifusion in Ld phases and only 11 ± 3% direct full fusion and 13 ± 4% hemifusion of HIV virions in the Lo phase domains (Fig. 5H). In contrast, HIV virions fused very efficiently (56 ± 3% of all particles) at the Lo/Ld domain boundaries and an additional 9 ± 4% hemifused in these locations (Fig. 5H). These results are qualitatively similar to but quantitatively more robust than those obtained in GPMVs and therefore validate SPPMs as a useful additional system to quantitatively analyze fusion in heterogeneous biological membranes at the single-particle level.

**DISCUSSION**

Membrane fusion in HIV entry is a highly cooperative process that must overcome several energy barriers associated with different fusion intermediates. This energy must be provided and released at the right time and at the right place to drive the merger between the viral and target membranes. Although cholesterol-rich lipid domains have been implicated in cell entry, their proposed involvement was puzzling because ordered lipids are thought to be detrimental to membrane bending and fusion. However, we recently discovered that Lo/Ld phase boundaries facilitate fusion in model systems and demonstrated that line tension at these boundaries might provide an additional previously unrecognized driving force for fusion (23). This previous work was limited because no receptors or co-receptors were present and because it could be argued that pure lipid model membranes oversimplify biological membranes. To overcome these limitations, we demonstrated in this work that a very similar mechanism governs HIV fusion with biological host membranes containing its natural receptors. We directly observed that lipid domain boundaries are the portals for HIV binding and membrane fusion, presumably because they present the weakest points in the host membrane. On the basis of these results, we propose the following model: HIV gp120 binds to CD4 receptors located in cholesterol-rich lipid domains, which exist transiently in the plasma membranes of living cells. If located near CCR5 co-receptors at domain boundaries, this initial binding leads to structural changes of gp120 and binding to the CCR5 co-receptor, enriching bound virions at the boundaries (Fig. 6A). After CD4 and CCR5 binding, gp41 changes its conformation to expose the fusion peptide to lipids in the boundary region (16). The predisposed preference of CCR5 for membrane domain boundaries greatly facilitates the targeting of the fusion peptide to these same fusion-promoting areas of the host membrane. Although we did not examine the other HIV co-receptor, CXCR4, in this study,
it might function equivalently to CCR5 in recruiting virions to domain boundary regions for more efficient fusion. Because receptors cooperate with membrane domain boundaries to form HIV entry sites, it would be interesting to examine in future studies the binding and fusion efficiencies of HIV particles with GPMVs or SPPMs, where CD4 and CCR5 are distributed differently or randomly. In contrast to many other enveloped viruses, HIV displays only a few (~14) trimeric glycoprotein spikes on its envelope (36). This small number is certainly inefficient for HIV entry and fusion. However, the cooperativity of HIV recognition and fusion at membrane domain boundaries may be a unique way to overcome this deficiency and may provide enough energy to drive fusion through its transition states with low Env copy numbers. Just as in model systems, we have shown here that line tension may also be a dominant force that promotes membrane fusion in biological membranes. The boundaries of the larger lipid domains in activated T cells could contribute to the elevated fusogenic capacity of activated T cells versus resting T cells. It is possible that activation of the immune system against other invading pathogens makes the cells more prone to HIV entry through the described mechanism. Therefore, enhanced domain activation could lead to an elevated production of HIV in people infected by other pathogens and thus an acceleration of the progression of AIDS (Fig. 6D).

Finally, this work also showed that GPMVs and a novel procedure to produce supported planar GPMV-derived membranes that we call SPPMs are very useful to study the selected targeting and membrane fusion of HIV virions. We anticipate that these methods will also be useful to study the binding, fusion, and properties of many other particles on cell-derived plasma membranes. The new approaches are versatile and should lead to many new discoveries regarding the role of lipid and protein heterogeneity of cell membranes. They may also serve as novel platforms for screening of viral entry inhibitors.

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**Fig. 4. Effect of lysosphingolipids and lysophospholipids on domain size and shape in GPMVs and their fusion with HIV Env particles.** (A) Influence of lysoPC and lysoSM on lipid phases of CD4+/CCR5+ GPMVs stained with Dil. Images were taken after treatment with lysoPC (20 µM) or lysoSM (5 µM) for 30 min at room temperature. The process is shown in movie S7. (B) Effect of lysoPC and lysoSM on binding and lipid mixing between HIV and GPMVs. The extent of HIV binding to GPMVs (n ≥ 25) was quantified as the number of HIV Env particles labeled with mKO-Gag on GPMVs at 4°C for 60 min after treatment with lysoSM or lysoPC. (C) Influence of PLA2 or SCDase treatment on lipid phases of GPMVs. Images were taken after treatment with PLA2 (10 U) or SCDase (10 mU) for 30 min at room temperature. Note that lysosphingolipids and lysophospholipids can be generated by PLA2 and SCDase, respectively. (D) Effect of PLA2 and SCDase on binding and lipid mixing between HIV and GPMVs. (E) Influence of lysolipids on lipid phases of GUVs. LysoPC (20 µM) or lysoSM (5 µM) was added to GUVs composed of brain SM (bSM)/brain PC (bPC)/brain phosphatidylserine (bPS)/cholesterol (2:1:1:1). The process is shown in movie S8. (F) Effect of lysolipids on the extent of liposome fusion mediated by the HIV fusion peptide. Liposomes composed of b5SM/b5PC/b5PS/cholesterol (2:1:1:1) and fusion peptide were added to liposomes preincubated with varying concentrations of lysolipids for 1 hour at room temperature. Scale bars, 10 µm. Data are means ± SEM (n = 3).
**Fig. 5. Binding and fusion of HIV Env particles to SPPMs.**

(A) Schematic diagram illustrating the formation of SPPM with ordered and disordered lipid domains (left) and TIRF microscopy setup to visualize the interaction of HIV with SPPM (right).

(B) Lateral distribution of CD4 and CCR5 in SPPM. Ld and Lo domains are visualized in SPPM by labeling the membranes with DiI and cholera toxin B (CTxB), respectively. SPPM colabeled with anti-CD4 (Alexa Fluor 488) and anti-CCR5 (Alexa Fluor 647) antibodies.

(C) Binding of HIV Env and VSV-G particles to SPPM. SPPM labeled with DiO (left) and bound HIV Env or VSV-G particles labeled with mKO-Gag (center) were visualized by epifluorescence and TIRF microscopy, respectively. Images of membrane-bound pseudoviruses were taken after 60 min of incubation at room temperature. The overlay image (right) shows the preferential binding of HIV but not VSV-G particles to the domain boundaries.

(D) Distribution of membrane-bound HIV Env and VSV-G particles on SPPM. Membrane-bound particles were analyzed for their distribution between three membrane regions (Ld, Lo, and a 0.75-μm-wide boundary region) of SPPM. Data are means ± SD (n = 3).

(E) CD4- and CCR5-dependent HIV binding. SPPMs were prepared with GPMVs isolated from CD4+/CCR5+ HeLa cells before and after treatment with MβCD and plain CD4−/CCR5− HeLa cells. Time courses of mean fluorescence intensities were recorded by TIRF microscopy measuring the binding of HIV Env particles to SPPM. Data are means ± SD (n = 3). (F) Fusion of HIV Env particles at Lo/Ld boundaries in an SPPM. Lipid phase separation in the SPPM was visualized by labeling with DiI, and fusion events of individual HIV Env particles labeled with lipid dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) were monitored by TIRF microscopy. HIV particles (green) fused with SPPM (red), causing round domains to show up because of DiD diffusion within the Lo phase. See also movie S9.

(G) Three characteristic types of events of single HIV Env particles on SPPMs. Representative fluorescence traces of single HIV Env particles including docking (constant fluorescence after initial binding), hemifusion (fluorescence decay to approximately half of the original), and full fusion (fluorescence decay to approximately baseline). The inset shows TIRF microscopy images of single particles after given times (in seconds) for each type of behavior. Time zero is defined as the first frame with a visible particle.

(H) Relative frequencies of single HIV fusion events in different regions of the SPPM. A total of 211 particles (31 on Ld, 128 on boundary, and 52 on Lo) were analyzed for their fusion events. Data are means ± SD (n = 3). Scale bars, 10 μm.
was removed by washing, and cells were returned to regular growth transfection. After 4 hours of incubation at 37°C, unincorporated dye (Gibco Life Technologies) containing 10
DiD-labeled HIV particles, 293T medium was replaced with Opti-MEM I centrifuged at 2500 rpm for 10 min at 4°C to clear debris. To prepare containing medium was collected at 48 hours after transfection and Env plasmids using the PolyFect reagent (Qiagen) (packaging construct), 1
293T cells with 3
containing MLV core proteins were prepared by cotransfection of HIV Env particles pseudotyped with envelope gp160 protein and HIV pseudovirus production
Virginia). GPMVs were produced and isolated from the cells, as previously described (17, 18), with slight modifications. Briefly, CD4+/CCR5+ HeLa cells were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum (FBS), G418 (0.2 mg/ml), 1% penicillin-streptomycin (PenStrep), and puromycin (1 µg/ml) in an incubator at 37°C with 5% CO2. Plain (CD4+/CCR5+) HeLa cells, which lack CD4 and CCR5, were grown in Dulbecco’s modified Eagle’s medium, 10% FBS, and 1% PenStrep in the same incubator. After growing the cells to a confluence of 70 to 90% in culture dishes, cells were washed three times with bleeding buffer [10 mM Hepes, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4)]. Membrane vesiculation was then induced by adding 25 mM formaldehyde and 2 mM DTT or 2 mM NEM in medium. After 24 hours of incubation, the extracellular medium was collected, centrifuged at low speed to remove cell debris, filtered through a 0.45-µm syringe filter, and stored at −80°C until use.

Preparation of GPMVs derived from CD4+/CCR5+ HeLa cells

CD4+/CCR5+ HeLa cells were provided by D. M. Rekosh (University of Virginia). GPMVs were produced and isolated from the cells, as previously described (17, 18), with slight modifications. Briefly, CD4+/CCR5+ HeLa cells were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum (FBS), G418 (0.2 mg/ml), 1% penicillin-streptomycin (PenStrep), and puromycin (1 µg/ml) in an incubator at 37°C with 5% CO2. Plain (CD4+/CCR5+) HeLa cells, which lack CD4 and CCR5, were grown in Dulbecco’s modified Eagle’s medium, 10% FBS, and 1% PenStrep in the same incubator. After growing the cells to a confluence of 70 to 90% in culture dishes, cells were washed three times with bleeding buffer [10 mM Hepes, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4)]. Membrane vesiculation was then induced by adding 25 mM formaldehyde and 2 mM DTT or 2 mM NEM in

Fig. 6. HIV enters cells at the boundaries of ordered lipid domains. (A) Schematic showing the sequential interaction of HIV gp120/gp41 with the CD4 receptor and CCR5 co-receptor and fusion peptide insertion at phase boundaries in heterogeneous cell membranes with ordered and disordered lipid domains. (B) Effect of cholesterol-rich lipid domains and their size on HIV membrane fusion. The recognition of HIV at domain boundaries leads to membrane fusion at these sites, where increased domain size with increased line tension lowers the energy barrier for fusion. (C) Coalescence and decomposition of dynamic lipid domains. Ordered lipid domains (red) in the plasma membranes of T cells are clustered by pathogen infection, whereas large domains are decomposed by lysosphingolipids. (D) Speculative model for HIV infection including the activation of CD4+ T cells and immune responses to invading pathogens. CD4+ T cells use ordered lipid domains for signal transduction against invading pathogens, whereas HIV uses the domains for entry into the cells. General immune responses after infection of pathogens are indicated by black arrows. Ordered membrane domains in resting T cell plasma membranes are nanoscopic and short-lived, but small dynamic domains can coalesce to create larger ones to function as signaling platforms upon pathogen invasion. Helper CD4+ T cells recognize the pathogen-derived antigens on the surface of antigen-presenting cells (APC) and become activated by coalescence of membrane domains. The activation of CD4+ T cells stimulates the ability of B cells and CD8+ T cells to defend against the invading pathogens. In this model, we propose that CD4+ T cells that are challenged by pathogens are more prone to HIV infection than resting T cells, as indicated by the red arrows. The HIV infection leads to apoptotic T cell death and ultimately results in the progression to AIDS.

MATERIALS AND METHODS

Materials

Most lipids and fluorescent probes were from Avanti Polar Lipids, and DiO, Dil, DiD, and octadecyl rhodamine B chloride (R18) were from Invitrogen. MβCD, NEM, and fluorescein isothiocyanate−, Alexa 647−, or Alexa 555−labeled CTxB were purchased from Sigma-Aldrich. Formaldehyde and DTT were purchased from Avantor and RPI, respectively. Alexa 488−labeled CD4 antibody and Alexa 647−labeled CCR5 antibody were purchased from Novus Biologicals. HIV entry inhibitors (maraviroc and enfuvirtide), SCDase, and PLA2 were purchased from Sigma-Aldrich. HIV fusion peptide labeled CCR5 antibody were purchased from Novus Biologicals.

HIV entry inhibitors (maraviroc and enfuvirtide), SCDase, and PLA2 were purchased from Sigma-Aldrich. HIV fusion peptide was custom-synthesized by the Yale W.M. Keck Biotechnology Resource Laboratory.

HIV pseudovirus production

HIV Env particles pseudotyped with envelope gp160 protein and containing MLV core proteins were prepared by cotransfection of 293T cells with 3 µg of pFB-Luc (MLV vector plasmid), 2 µg of pHIT60 (packaging construct), 1 µg of MLV mKO-Gag, and 3 µg of HIV JRFL Env plasmids using the PolyFect reagent (Qiagen) (16). The virus-containing medium was collected at 48 hours after transfection and centrifuged at 2500 rpm for 10 min at 4°C to clear debris. To prepare DiD-labeled HIV particles, 293T medium was replaced with Opti-MEM I (Gibco Life Technologies) containing 10 µM DiD at 16 to 20 hours after transfection. After 4 hours of incubation at 37°C, unincorporated dye was removed by washing, and cells were returned to regular growth medium. After 24 hours of incubation, the extracellular medium was collected, centrifuged at low speed to remove cell debris, filtered through a 0.45-µm syringe filter, and stored at −80°C until use.

For control experiments, MLVs pseudotyped with VSV-G and MLV core proteins were also prepared. For bulk binding and lipid mixing assays, membranes of viral particles were labeled with 20 µl of octadecyl rhodamine B chloride (1 mg/ml; R18) in 50 µl of virus for 60 min at room temperature and then free R18 was removed on a Sephadex PD-10 desalting column (GE Healthcare). The infectious titer was determined by a luciferase assay, as previously described (42), in CD4+/CCR5+ HeLa cells for HIV Env pseudotyped viruses.
blebbing buffer for 1 hour at 37°C. Following shaking, solutions containing cell-detached GPMVs were gently decanted into a 50-mL tube. The GPMVs were placed on ice for 30 min to settle relatively large GPMVs (~10 μm in diameter), which were then transferred from the bottom of the tube to coverslips for observation on an epifluorescence microscope.

**Lateral distribution and relative amount of CD4 and CCR5 on GPMVs**

We observed the lateral distributions of CD4 and CCR5 on cell-attached and isolated GPMVs. For the preparation of cell-attached GPMVs, CD4+/CCR5+ HeLa cells were seeded on quartz slides or 18-mm glass coverslips and grown to about 40% confluence. After inducing GPMVs from the cells, as described above, they were rinsed extensively with phosphate-buffered saline (PBS) to remove GPMV-inducing chemicals. Cell-attached GPMVs or isolated GPMVs were blocked with 0.1% bovine serum albumin in PBS and then the vesicles were stained with the LD marker Dil (0.2 μM) or DiO (0.5 μM) and/or the Lo marker Alexa 555-conjugated CTxB (1 μg/mL) for 60 min on ice. After labeling, the GPMVs were incubated with an anti-CD4 antibody and/or an anti-CCR5 antibody conjugated with Alexa 488 and/or Alexa 647, respectively, at a concentration of 5 μg/mL at 4°C for 60 min. The lateral distribution of fluorescent lipids and proteins on cells or GPMVs was visualized using an epifluorescence microscope. The immunofluorescence staining was also used to evaluate the amount of CD4 and CCR5 on the surface of GPMVs. For quantification, isolated GPMVs were incubated with antibodies at 4°C for 60 min and then unbound antibodies were removed by centrifugation (16,000g) for 30 min at 4°C. GPMV pellets were resuspended in 400 μL of Hepes buffer. Relative amounts of CD4 and CCR5 were measured at room temperature by fluorescence emission intensities of Alexa 488 at 520 nm and Alexa 647 at 667 nm in a Jobin-Yvon Fluorolog-3 spectrophotometer (Jobin-Yvon) with excitation and emission wavelengths of 555 and 590 nm, respectively. Dequenching of R18 fluorescence was normalized to the initial fluorescence (F/F0).

**Lipid mixing between HIV and GPMVs**

Fusion between R18-labeled virus particles and unlabeled GPMVs was measured by the dequenching of R18 fluorescence. Unlabeled GPMVs were added to the R18-labeled virions (1 × 10^8 particles) in Hepes buffer at room temperature. Concentration of GPMVs was estimated by total protein concentration in GPMVs using a bicinchoninic acid assay. Protein concentrations of GPMVs derived from CD4+/CCR5+, MβCD-treated CD4+/CCR5+, and plain CD4+/CCR5+ HeLa cells were 101, 87, and 62 μg/mL, respectively. Fluorescence intensities were measured under constant stirring using a Fluorolog-3 spectrofluorometer (Jobin-Yvon) with excitation and emission wavelengths of 555 and 590 nm, respectively. Dequenching of R18 fluorescence was normalized to the initial fluorescence (F/F0).

**Electron cryo-microscopy**

To prepare the samples for electron cryo-microscopy (cryoEM), HIV Env particles were incubated with GPMVs for 60 s at room temperature to capture early stages of membrane fusion between HIV particles and GPMVs. Samples (~3.5 μL) were applied to either c-flat or quantifoil holey carbon grids, manually blotted to near dryness with filter paper, and rapidly plunged into a slurry of liquid ethane. The grids were then transferred under liquid nitrogen to a Tecnai F20 cryoEM (Philips/FEI) operating at 120 kV. Images were recorded at magnifications of ×11,000 or ×30,000 under low-electron dose conditions (~20 e^-/Å^2) using a 4k × 4k charge-coupled device (CCD) camera (Gatan).

**Treatment of GPMVs with MβCD and lysolipids**

For cholesterol depletion, isolated GPMVs from CD4+/CCR5+ HeLa cells were incubated with 5 mM MβCD in Hepes buffer for 30 min at room temperature. Other GPMVs were incubated with 5 μM lysoSM or 20 μM lysoPC in Hepes buffer for 30 min at room temperature to examine the effect of lysolipids on lipid phases. Yet, other GPMVs were added to 10 U of PL2A in 10 mM tris-HCl buffer (pH 8.5) or 10 μM of SCDase in 50 mM sodium acetate buffer (pH 6.0), which was adjusted to an osmolality of 300 mmol/kg by the addition of NaCl, and incubated at room temperature for 30 min. After incubation, GPMVs were isolated by centrifugation (16,000g) for 30 min at 4°C and were used for measurements of HIV binding and fusion.

**Fluorescence imaging**

Fluorescence images were recorded on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss) with a mercury lamp as a light source, 40x or 63x water immersion objectives (Carl Zeiss; numerical aperture, 0.95), and an electron-multiplying CCD cooled to −70°C (iXon DV887ESC-BV, Andor) as a detector. Images were acquired using homemade software written in LabVIEW (National Instruments). Membranes stained with DiO or NBD were epi-illuminated through a 480-nm band-pass filter (D480/30, Chroma) and via a dichroic mirror (505dclp, Chroma). Fluorescence was observed through a 535-nm band-pass filter (D535/40, Chroma), Dil- or rhodamine-stained membranes and mKO-labeled viral particles were epi-illuminated through a 540-nm band-pass filter (D540/25, Chroma) and via a dichroic mirror (565dclp, Chroma). Fluorescence was observed through a 665-nm band-pass filter (D665/55, Chroma). Viral particles or LUVs stained with DiD were illuminated through a 620-nm band-pass filter (ET620/60, Chroma) and observed through a 665-nm band-pass filter (HQ665/60, Chroma). All fluorescence imaging was performed at room temperature (~22°C). Images were processed and assessed for colocalization using ImageJ.
Preparation of GUVs

GUVs were prepared via the electroformation technique. Briefly, 25 μl of a 10 mM lipid mixture composed of bSM/bPC/bPS/cholesterol (2:1:1:1) containing the fluorescent lipid probe Rh-PE (0.1 mole percent (mol %)) was deposited on the conducting surfaces of two indium tin oxide–coated glass slides. After the slides were placed in a vacuum desiccator for 90 min to remove residual solvent, a fabricated chamber composed of two conducting slides facing each other separated by a 0.5-mm spacer was filled with 300 mM sucrose in H₂O. GUVs were generated by applying alternating electric current (3 V, 10 Hz) through a function generator for 120 min at 60°C and transferred into a 300 mM glucose solution to set by gravity on the microscope slide.

Lipid mixing of LUVs mediated by HIV fusion peptide

LUVs were prepared by extrusion. Phospholipids composed of bSM/bPC/bPS/cholesterol (2:1:1:1) were dissolved in a mixture of chloroform and methanol, and the solvent was evaporated under a stream of nitrogen gas in a glass test tube. The thin lipid film was further dried overnight under vacuum and hydrated with 0.5 ml of Hepes buffer [10 mM Hepes and 120 mM NaCl (pH 7.2)]. The suspension was vigorously vortexed for 5 min, was subjected to 10 freeze-thaw cycles, and was then extruded 21 times through two stacked polycarbonate filters with a pore size of 100 nm (Avestin). The lipid mixing assay was based on fluorescence resonance energy transfer between NBD-PE and rhodamine-PE. The HIV fusion peptide was added to 50 μM LUVs with a ratio of 1:9 of labeled (1 mol % of NBD-PE and rhodamine-PE each) to unlabeled LUVs in Hepes buffer at room temperature. The fluorescence was recorded under constant stirring in a Fluorolog-3 spectrofluorometer (Jobin-Yvon) with the excitation and emission wavelengths set at 460 and 535 nm, respectively. The value for 0% lipid mixing was the fluorescence intensity of the LUV suspension before the fusion peptide was added, whereas the value for 100% lipid mixing was obtained by adding Triton X-100 [final concentration, 0.5% (v/v)] to the suspension at the end of each experiment.

Preparation of SPPMs

Quartz slides were cleaned by boiling in Contrad detergent for 15 min and by sonication in a hot bath for 30 min. After rinsing with deionized water and ethanol, the slides were immersed in Piranha solution (3:1 mixture of 95% sulfuric acid and 30% hydrogen peroxide) to remove water and ethanol, the slides were immersed in Piranha solution (3:1 mixture of 95% sulfuric acid and 30% hydrogen peroxide) to remove remaining organic residues, followed by extensive rinsing in pure water. The first leaflet of the SPPM was prepared by Langmuir-Blodgett (LB) transfer directly onto the quartz slide. Lipid mixtures composed of bSM/bPC/cholesterol (2:2:1) with 3 mol % of DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine)–polyethyleneglycol–triethoxysilane (43) were spread onto a pure water surface in a NIMA 611 LB trough (KSV NIMA). After allowing the solvent to evaporate for 10 min, the monolayer was compressed at a rate of 10 cm²/min to reach a surface pressure of 32 mN/m. Immediately before use, quartz slides were further cleaned for 10 min in an argon plasma sterilizer (Harrick Scientific) and then dipped into the trough at a speed of 200 mm/min and withdrawn at 5 mm/min while keeping the monolayer surface pressure constant at 32 mN/m. The transferred lipid monolayer was dried in a vacuum desiccator at room temperature overnight and cured in a 70°C oven for 40 min to covalently link the polymer to the SiO₂ slide surface. After equilibration in the desiccator to room temperature, the slide with the tethered polymer-supported LB monolayer was placed in a custom-built flow-through chamber. A suspension of GPMVs in Hepes buffer was injected into the chamber and incubated for at least 2 hours at room temperature to form the distal leaflet of the SPPM bilayer. Excess unfused GPMVs were then washed out by extensive rinsing with Hepes buffer.

Fluorescence recovery after photobleaching

The integrity of the SPPMs and the diffusion of the lipids within them were examined by fluorescence recovery after photobleaching (FRAP). Bilayers were bleached in a pattern of parallel stripes, and the data were fit to the model $F(t) = F_\infty + (F_0 - F_\infty)e^{-(D/t)}$, where $F_0$ and $F_\infty$ are the initial and final fluorescence intensities after bleaching, respectively, $a = 2\pi/p$, $p$ is the stripe period (12.7 or 3.2 μm), and $D$ is the lateral diffusion coefficient. The mobile fraction (m), which reflects the percentage of observed fluorescence recovery within the time frame of a FRAP experiment (<1 min), is given by $m_f = \frac{F_{\infty} - F_0}{F_{\infty} - F_0} \times 200$, where $F_{\infty}$ is the fluorescence intensity before photobleaching. Ten regions on three independently prepared bilayers were sampled to determine the reported average values.

Binding and fusion of single HIV Env particles on SPPMs by TIRF microscopy

To measure binding to SPPMs, mKO-labeled (mKO-Gag) HIV Env particles were injected into a chamber with unlabeled SPPMs, and the fluorescence increase by HIV binding to SPPMs was monitored over time by prism-based TIRF microscopy. The prism-quartz interface was lubricated with glycerol to allow easy translocation of the sample cell on the microscope stage. The light source was an argon ion laser (Innova 90C, Coherent) emitting light at 514 nm, and fluorescence was observed through a 610-nm band-pass filter (D610/60, Chroma). The beam was totally internally reflected at an angle of 72° on the normal to produce an evanescent wave. The intensity of the laser beam was computer-controlled through an acousto-optic modulator (Isomet) or could be blocked entirely by a computer-controlled shutter. The laser intensity, shutter, and camera were controlled by a homemade program written in LabVIEW (National Instruments). To investigate the targeting of HIV particles to different regions of Ld/Lo phase-separated SPPMs, mKO-labeled particles were incubated with SPPMs that were stained with DiO for 60 min at room temperature. After unbound particles were washed away with Hepes buffer, phase-separated SPPMs were visualized by epifluorescence microscopy, and bound particles were visualized by TIRF microscopy. To analyze and quantify the distribution of SPPM-bound particles, we distinguished three regions of the SPPM: Lo domains, Ld phase areas, and Lo/Ld boundary regions with a 0.75-μm width centered on the perimeter of each Lo domain. Custom-built particle-tracking software (35) was used to automatically detect the position (x and y coordinates) of each particle. To monitor the fusion of single HIV particles with the SPPM, DiD-labeled HIV Env particles were injected into a chamber, one large window wall of which was formed by the SPPM. The light source for TIRF illumination was a diode laser (CUBE640, Coherent) to excite the lipid dye DiD through a 620-nm filter (ET620/60, Chroma) and via a dichroic mirror (660dclp, Chroma). DiD fluorescence was observed through a 665-nm long-pass filter (HQ665/60, Chroma). Data acquisition and image analysis were accomplished through custom-built software written in LabVIEW (National Instruments) (35). Single events that included docking, hemifusion, and full fusion were measured by analyzing the peak fluorescence intensities from each bound particle as a function of time: Events with no fluorescence change over time were classified as docking, those with decays to around one-half of the original peak intensity were classified as hemifusion, and those with complete decays were classified as direct full fusion events (44).
**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/6/e1700338/DC1

fig. S1. Association of CD4 and CCR5 with lipid rafts in CD4+/CCR5+ HeLa cells with stably expressed CD4 and CCR5.

fig. S2. Formation of GPMVs by treatment of CD4+/CCR5+ HeLa cells with small amounts of formaldehyde and DTT.

fig. S3. Partitioning of CD4 and CCR5 in GPMVs induced by NEU instead of formaldehyde and DTT from CD4+/CCR5+ cells.

fig. S4. HIV Env pseudovirus particles bind preferentially at boundaries between coexisting Lo and Ld domains in GPMVs.

fig. S5. VSV-G pseudovirus particles bind to Lo membrane regions in GPMVs.

fig. S6. Electron cryo-micrographs of HIV Env particles bound to GPMVs.

fig. S7. Modulation of lipid phases does not affect binding of HIV to GPMVs.

fig. S8. Preparation of SPPMs with GPMVs.

fig. S9. Lateral distribution of CD4/CCR5 in SPPMs.

fig. S10. Formation of GPMVs by treatment of CD4+/CCR5+ HeLa cells with small amounts of formaldehyde and DTT.

**REFERENCES AND NOTES**


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HIV virions sense plasma membrane heterogeneity for cell entry
Sung-Tae Yang, Alex J. B. Kreutzberger, Volker Kiessling, Barbie K. Ganser-Pornillos, Judith M. White and Lukas K. Tamm

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