Viruses acquire their envelope by budding from a host cell membrane, but viral lipid composition may differ from that of the budding membrane. We have previously reported that the HIV-1 membrane is highly enriched in cholesterol, sphingolipids, and other raft lipids, suggesting that the virus may bud from pre-existing or virus-induced lipid rafts. Here, we employed the environmentally sensitive fluorescent dye Laurdan to study the membrane lateral structure of HIV-1 derived from different cell lines. Differences in viral membrane order detected by Laurdan staining were shown to be due to differences in lipid composition. Isogenic viruses from two different cell lines were both strongly enriched in raft lipids and displayed a liquid-ordered membrane, but these effects were significantly more pronounced for HIV-1 from the T-cell line MT-4 compared with virus from 293T cells. Host-dependent differences in the lipidome predominantly affected the ratio of sphingomyelins (including dihydrosphingomyelin) to phosphatidylcholine, whereas cholesterol contents were similar. Accordingly, treatment of infectious HIV-1 with the sphingomyelin-binding toxins Equinatoxin-II or lysenin showed differential inhibition of infectivity. Liposomes consisting of lipids that had been extracted from viral particles exhibited slightly less liquid order than the respective viral membranes, which is likely to be due to absence of membrane proteins and to loss of lipid asymmetry. Synthetic liposomes consisting of a quaternary lipid mixture emulating the viral lipids showed a liquid order similar to liposomes derived from virion lipids. Thus, Laurdan staining represents a rapid and quantitative method to probe viral membrane liquid order and may prove useful in the search for lipid active drugs.

HIV-1 is an enveloped retrovirus, which acquires its lipid envelope by budding from the plasma membrane of the infected host cell. Several reports have shown that the viral membrane is enriched in sphingomyelin (SM), including the unusual sphingolipid dihydrosphingomyelin (DHSM) and collectively referred to as sphingomyelins (SMs), glycosphingolipids, cholesterol (CHOL), saturated phosphoglycerolipids and phosphoinositides (1–4). Moreover the CHOL/phospholipid and protein/lipid ratios of the HIV-1 membrane are high, corresponding to a highly ordered membrane, and are presumed to be different from the overall host cell plasma membrane. Accordingly, the HIV-1 envelope has been considered to be a large raft-like membrane microdomain (3). This is in line with previous reports describing enrichment of raft markers in the HIV-1 membrane and its sensitivity to CHOL-depleting agents (5–9). Furthermore, HIV-1 glycoproteins have been suggested to localize within membrane rafts due to palmitoylation of two cysteines (10), and the main structural Gag protein has been shown to rapidly relocalize to detergent-resistant membranes after initial membrane binding (6).

Membrane microdomains are dynamic assemblies resulting from the lateral interaction of lipids and proteins. Two phases coexist in the plasma membrane: the liquid-ordered phase (l_o), mainly composed of CHOL and sphingolipids (SPLs), and the liquid disordered phase (l_d), mainly composed of glycerophospholipids (11–13). In the activated state, l_o microdomains can coalesce and serve as platforms for membrane trafficking, signaling, and virus budding (14, 15). The first method to biochemically enrich membrane rafts was the purification of detergent-resistant membranes, based on their resistance to extraction with non-ionic detergent at 4 °C (16). However, this and other methods based on antibody or cholera toxin binding may lead to artificial aggregation of membrane microdomains and thus do not necessarily represent their native state (17, 18). For these reasons and because the association and dissociation of membrane microdomains appears to occur on a rapid timescale and the raft size is too small to be optically resolved, the
raft concept remains controversial. However, the determination of the HIV-1 lipidome, a native membrane purified without any detergent, has provided strong evidence for the existence of these microdomains (3).

Fluorescent lipid analogs that partition preferentially into a specialized lipid phase could be an attractive tool to study membrane microdomains. However, partitioning of such dyes mainly depends on the local chemical environment and not on the phase state of the membrane (19–21). In contrast, Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) is a lipophilic dye that binds to membranes independent of their phase state but reports the phase state by a change in its fluorescence emission (20). Laurdan exhibits a blue shift in its emission spectrum with increasing membrane condensation. This is caused by an alteration in the dipole moment of the probe as a consequence of exclusion of water molecules from the lipid bilayer. Thus, excitation of membrane bound Laurdan leads to two emission maxima representing differences in membrane lateral structure. Quantification of membrane order is achieved by computing the Generalized Polarization (GP) value, which is defined as normalized intensity ratio of the two emission channels. GP values range from +1 (most condensed) to −1 (most fluid). They are not biased by probe concentration, membrane ruffles, and surface modifications, such as lipoprotein binding. Furthermore, there is no preferential interaction with a specific lipid, fatty acid, or head group (20, 21). GP value correspondance to different lipid phases was estimated using liposomes with a composition similar to that of cellular membranes (22, 23). Using an equimolar mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine, CHOL, and SM as an \( l_o \) membrane, and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) as an \( l_d \) membrane and solid ordered \( (s_o) \) phase, GP values below +0.25 were shown to correspond to the \( l_d \) phase, GP values between +0.25 and +0.5 to the \( l_o \) phase, and GP values above +0.5 to the \( s_o \) phase (22, 23).

Laurdan has been extensively used to characterize domain formation and lateral lipid segregation in model membranes composed of different phospholipid mixtures or lipids extracted from cellular membranes (19, 22–25). It has also been used to study the membrane structure in living cells. Gaus and coworkers observed \( l_o \) domains enriched on membrane protrusions (filopodia), adhesion points, and cell-cell contacts (26). They also used Laurdan to address the physical properties of the plasma membrane around the T-cell receptor in activated T cells, observing larger and more stably ordered membrane domains at sites of T-cell activation (27). Quantitative determination of cellular plasma membrane order by fluorescence spectroscopy is complicated due to the rapid internalization and redistribution of the probes to other cellular membranes, making it difficult to interpret the fluorescence measurements over the whole cell. This problem is not encountered in purified virus particles, because they contain only a single membrane. We therefore developed an assay to study virally labeled structure by fluorescence spectroscopy. For this purpose, isogenic HIV-1 particles were produced in two different cell lines, and their GP profiles were determined. In parallel, the lipid constituents were quantified by mass spectrometry. The viral membrane displayed a \( l_o \) structure in both cases, but this was more prominent for the virus derived from the T-cell line MT-4 compared with virus derived from 293T cells. The validity of this result was supported by comparing the lipidome of the two viruses, which revealed a significantly higher SMs/phosphatidylcholine (PC) ratio for the MT-4-derived virus. Accordingly, treatment with SM-binding toxins inactivated MT-4-derived virus more efficiently than 293T-derived virus, whereas both viruses exhibited similar infectivities before treatment. The reported approach allows rapid determination of differences in viral membrane order, permitting screening for compounds that perturb \( l_o \) domains, which may act as antivirals of a novel type.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Virus Purification**—293T cells and TZM reporter cells (JC53BL cells expressing β-galactosidase and luciferase under the control of the HIV-1 LTR) (28) were kept in Dulbecco’s modified Eagle’s medium, MT-4 cells (29), and C8166 cells (30) were kept in RPMI 1640 medium. Both media were supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, 4 mM glutamine, and 10 mM Hepes. Cell cultures were maintained at 37 °C and 5% CO₂. For virus production, MT-4 cells were infected with HIV-1 strain NL4-3 (31), and virus was harvested from cocultures of infected, and uninfected cells before cytopathic effects were observed (32). 293T cells were transfected with the isogenic proviral plasmid pNL4-3 by calcium phosphate precipitation. For generation of pseudotyped particles, cells were cotransfected with a plasmid expressing the G glycoprotein of vesicular stomatitis virus (VSV-G (33)) together with pNL4-3 or ΔEnvNL4-3, kindly provided by V. Bosch (Deutsches Krebsforschungszentrum, Heidelberg) at a molar ratio 1:2. Fluorescence resonance energy transfer reporter particles were generated by cotransfection of plasmid pCHIV (34) and its derivatives carrying a fluorescent moiety between the MA and CA domains of Gag, pCHIVeYFP and pCHIVeCFP, at a molar ratio of 2:1. HIV-1 purification was performed essentially as described (32, 35). Briefly, medium was harvested and cleared by filtration, and particles were concentrated by ultracentrifugation through a cushion of 20% (w/w) sucrose. Concentrated HIV-1 was further purified by velocity gradient centrifugation on an OptiPrep gradient (Axis-Shield, Oslo, Norway). The visible virus fraction was collected and concentrated by centrifugation. The final pellet was resuspended in 150 mM NaCl, 10 mM Hepes, pH 7.4, rapidly frozen in liquid nitrogen and stored at −80 °C. The particle concentration was determined by p24 enzyme-linked immunosorbent assay. Inactivation of infectious HIV-1 was performed by incubating the virus with 5 mM AT-2 (2,2’-dithiodipryidine, aldrithiol-2, Sigma) for 1 h at 37 °C with gentle stirring as described (36). Successful inactivation was controlled by culturing inactivated samples for 10 days with highly susceptible C8166 cells.

**Infectivity Assay**—To determine differences in infectivity, isogenic HIV-1NL4-3 was produced in parallel from MT-4 or 293T cells. Because 293T cells do not carry the viral receptor, both cell lines were infected with VSV-G-pseudotyped virus and progeny virus (lacking VSV-G) was analyzed. In a second experiment virus was produced from 293T cells by calcium
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phosphate precipitation. The respective viruses were titered in parallel on MT-4 or C8166 cells. C8166 cells were seeded in 96-well plates and infected with different virus dilutions. Infection was monitored by syncytia formation, and the tissue culture infectious dose 50% was determined after 7 or 8 days. MT-4 cells were seeded in 24-well plates and infected with different amounts of virus. 35 h post infection cells were harvested, fixed with 4% paraformaldehyde, and permeabilized. HIV-1 infected cells were identified by fluorescence-activated cell sorting analysis following staining with a phycoerythrin-conjugated antibody against the viral p24 capsid protein (KC57-RD1, Beckman Coulter, Inc., Fullerton, CA). The percentage of phycoerythrin-positive cells was analyzed by FACSCalibur using BD CellQuest Pro 4.0.2. Software (BD Pharmingen).

Laurdan Staining and Purification of Labeled Particles—Laurdan was dissolved in DMSO, and staining was performed on OptiPrep-purified HIV-1 particles, using between 3 and 7.5 μg of p24 per reaction. Viral particles were incubated for 10 min at room temperature with 5 μM Laurdan (Molecular Probes, Eugene, OR) under gentle stirring. Labeled particles were subsequently purified by ultracentrifugation for 45 min through a 20% sucrose cushion in a SW60 rotor at 44,000 rpm. Particles were carefully resuspended in 150 mM NaCl, 10 mM Hepes, pH 7.4, and analyzed by fluorescence spectroscopy.

In the case of Methyl-β-cyclodextrin (Mβ-CD, Sigma) treatment, Laurdan-labeled HIV-1 particles were incubated with 5 mM Mβ-CD at 37 °C for 30 min under gentle stirring. Subsequently, viral particles were collected by ultracentrifugation for 60 min in an SW-60 rotor at 22,000 rpm centrifuging through a 20% and onto a 70% sucrose cushion.

Fluorescence Spectroscopy and Determination of GP Values—All fluorescence measurements were made using an SLM Aminco series 2 (Spectronic Instruments, Rochester, NY) spectrofluorometer. Temperature was controlled during all measurements, and samples were allowed to equilibrate for 5 min before measurement at each temperature. Laurdan excitation was set at 355 nm, and emission spectra were recorded. The fluorescence intensity of solvent-treated unstained HIV-1 purified in parallel was subtracted to remove the signal due to particle scattering. Furthermore, the spectrofluorometer provides an internal instrument correction, which was applied in all measurements. The program corrects any wavelength-dependent instrument response.

To quantify changes in the Laurdan emission spectrum, GP values were calculated. In analogy with fluorescence polarization they are defined as: \( GP = (I_B - I_R)/(I_B + I_R) \), where \( I_B \) (at 440 nm) and \( I_R \) (at 490 nm) correspond to the intensities at the blue and red edges of the emission maxima, respectively (37, 38).

Virus Treatment with Toxins and Infectivity Assay—To investigate the inhibitory effect of Equinatoxin-II (Eqt-II) or lysenin on viral infectivity, purified HIV-1 particles were treated with toxins, and their infectivities were determined by titration on TZM cells. Eqt-II was purified as described previously (39), and lysenin was purchased from Peptide Institute (Osaka, Japan). OptiPrep-purified HIV-1 particles corresponding to 500 ng of p24 per reaction were incubated in a total volume of 20 μl in phosphate-buffered saline with varying amounts of Eqt-II or lysenin. Incubation was done for 20 min at room temperature for Eqt-II and for 30 min at 37 °C for lysenin. Subsequently, 180 μl of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was added to inactivate unbound toxin molecules. Toxin treatment of mock particles and subsequent inactivation was performed to rule out direct effects of the toxins on target cells. The infectivity of toxin-treated HIV-1 was determined on TZM reporter cells as described (40) with some modifications. TZM cells (8 × 10^3 cells/well) were seeded 1 day before infection in a 96 well plate and were infected with serial 5-fold dilutions of toxin-treated virus. At 48 h post-infection, cells were lysed and luciferase activity was measured in the lysates as described by the manufacturer using the Promega Steady Glo kit and a microplate luminometer (Luminoskan Ascent, Thermo Labsystems).

Production of Lipid Vesicles—Large unilamellar vesicles (LUV) were prepared following the extrusion method described by Mayer et al. (41). All lipids were from Avanti Polar Lipids (Birmingham, AL). Lipids were mixed in chloroform and dried under nitrogen. Traces of organic solvent were removed by vacuum pumping for 1–2 h. Subsequently, the dried lipid film was dispersed in 10 mM Hapes, 150 mM NaCl (pH 7.4) and subjected to 10 freeze-thaw cycles prior to extruding 10 times through two stacked polycarbonate membranes with a 100 nm pore size (Nucleopore, Inc., Pleasanton, CA) using the Thermo-barrel extruder (Lipex extruder, Northern Lipids, Inc., Burnaby, Canada). Distribution of vesicle sizes was estimated by quasielastic light scattering using a Malvern Zeta-Sizer instrument (Malvern Instruments, Malvern, UK).

Lipid Analysis—Lipid analysis was performed as described in Brügger et al. (3). Cholesterol quantification was performed employing a derivatization protocol described by Liebisch et al. (42). Cholesterol acetate was analyzed by nano-electrospray ionization-tandem mass spectrometry monitoring a neutral loss of m/z 77. Quantitation was done using d6-cholesterol as internal standard, which was likewise derivatized to d6-cholesterol acetate.

RESULTS

GP Values Derived from Laurdan Emission Spectra Report HIV-1 Membrane Phase State—Based on previous reports showing that the HIV-1 envelope is highly enriched in raft lipids (1–4), we used the polarity-sensitive dye Laurdan (38) to measure the lateral structure of HIV-1 membranes. Laurdan is homogeneously distributed within the membrane and has an emission maximum around 490 nm for fluid membranes and around 440 nm for condensed membranes (20). The phase state of a membrane can be quantified by the GP value, which is defined as the normalized intensity ratio of the two emission channels and provides a relative measure of lipid order.

HIV-1 particles lacking viral Env proteins and pseudotyped with VSV-G glycoprotein were used to establish the assay, because they result in high particle yields, permit titration of infectivity, and are of lower biohazard than infectious HIV-1. Purified particles were produced following transfection of 293T cells, and labeled with Laurdan as described under “Experimental Procedures.” Using single-round infectivity assays on TZM
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To validate that Laurdan emission spectra report membrane lateral structure, Mβ-CD was used to extract CHOL from the viral membrane, one of the main components of $l_c$ domains (11, 12). Laurdan-labeled particles were treated with 5 mM Mβ-CD, and emission spectra were determined as above (Fig. 1A, right panel). A prominent red shift was observed at all temperatures indicating increased membrane fluidity after CHOL extraction. To ensure comparability of results, Mβ-CD-treated and untreated particles were purified, stained, collected, and measured in parallel using an identical protocol (except for omission of Mβ-CD in the untreated sample). As previously described (7), Mβ-CD treatment was found to increase the buoyant density of viral particles. Viral integrity after Mβ-CD treatment was confirmed by comparing the protein pattern of Mβ-CD-treated and untreated particles on silver-stained gels (data not shown). Particle integrity was further analyzed using a fluorescence resonance energy transfer-based assay: Double-labeled HIV-1 particles were produced carrying cyan fluorescent protein or yellow fluorescent protein fused to the MA domains of part of their structural Gag molecules (40). Such particles exhibit a strong fluorescence resonance energy transfer signal, which was not affected by the procedure of Laurdan labeling. The fluorescence resonance energy transfer signal was largely retained upon treatment with 5 mM Mβ-CD, whereas it was instantly lost upon disruption of the viral membrane by detergent treatment (supplemental Fig. S1).

Membrane order was quantified by calculating GP values from the two spectra (Fig. 1B). At room temperature ($\sim 20 ^\circ$C), the GP value for the untreated viral membrane was +0.43 with $l_c$ membranes expected to display GP values between +0.25 and +0.5 (22, 23, 26, 27). GP values gradually decreased with increasing temperature. The temperature profile of GP values was very different for Mβ-CD-treated particles, which exhibited a GP value close to +0.25 at room temperature and a much steeper decline at rising temperature.

To analyze if VSV-G-pseudotyped particles accurately match native HIV-1 in membrane order, we compared VSV-G-pseudotyped HIV-1 Env proteins and wild-type HIV-1 virions regarding their GP profiles at different temperatures (Fig. 2A). Prior to Laurdan staining, infectious HIV-1 was inactivated with AT-2, which covalently modifies the essential zinc fingers in the viral nucleocapsid protein. This compound abolishes viral infectivity but does not appear to compromise the membrane protein structure (36). The GP profiles were virtually indistinguishable, suggesting that neither pseudotyping nor inactivation with AT-2 had an influence on membrane lateral structure. To check for an effect of inactivation, we com-

**FIGURE 1. Laurdan emission spectra and GP profile of HIV-1.** A, HIV-1 particles pseudotyped with VSV-G were purified from transfected 293T cells and stained with Laurdan as described under "Experimental Procedures." Particles were subsequently incubated with (right panel) or without (left panel) 5 mM Mβ-CD. Stained particles were excited at 355 nm in a spectrofluorometer at the indicated temperature, and emission spectra were recorded. B, GP values were calculated for each temperature from five independent experiments and are displayed for particles treated (solid line) or not treated (dashed line) with Mβ-CD. The $T_m$ is indicated by the arrow. Error bars represent standard deviation of the mean.

reporter cells, viral infectivity was found to be largely retained after Laurdan labeling (data not shown). Particles were incubated at increasing temperatures, excited at 355 nm, and emission spectra were recorded. Fig. 1A, left panel, shows emission spectra for Laurdan-labeled particles. At room temperature ($\sim 20 ^\circ$C) a strong signal was observed around 440 nm, which was gradually lost when the temperature was increased, concomitant with a red shift in emission. This indicates a progressive decrease in membrane order with rising temperature due to water dipolar relaxation (20, 21).

To ensure comparability of results, Mβ-CD treatment was found to increase the buoyant density of viral particles. Viral integrity after Mβ-CD treatment was confirmed by comparing the protein pattern of Mβ-CD-treated and untreated particles on silver-stained gels (data not shown). Particle integrity was further analyzed using a fluorescence resonance energy transfer signal, which was not affected by the procedure of Laurdan labeling. The fluorescence resonance energy transfer signal was largely retained upon treatment with 5 mM Mβ-CD, whereas it was instantly lost upon disruption of the viral membrane by detergent treatment (supplemental Fig. S1).

Membrane order was quantified by calculating GP values from the two spectra (Fig. 1B). At room temperature ($\sim 20 ^\circ$C), the GP value for the untreated viral membrane was +0.43 with $l_c$ membranes expected to display GP values between +0.25 and +0.5 (22, 23, 26, 27). GP values gradually decreased with increasing temperature. The temperature profile of GP values was very different for Mβ-CD-treated particles, which exhibited a GP value close to +0.25 at room temperature and a much steeper decline at rising temperature.

To analyze if VSV-G-pseudotyped particles accurately match native HIV-1 in membrane order, we compared VSV-G-pseudotyped HIV-1 Env proteins and wild-type HIV-1 virions regarding their GP profiles at different temperatures (Fig. 2A). Prior to Laurdan staining, infectious HIV-1 was inactivated with AT-2, which covalently modifies the essential zinc fingers in the viral nucleocapsid protein. This compound abolishes viral infectivity but does not appear to compromise the membrane protein structure (36). The GP profiles were virtually indistinguishable, suggesting that neither pseudotyping nor inactivation with AT-2 had an influence on membrane lateral structure. To check for an effect of inactivation, we com-
pared AT-2-inactivated complete HIV-1 with HIV-1-like particles produced by transfection of an expression vector (pCHIV), which encodes all viral proteins but lacks essential replication elements. These latter particles are non-infectious and were thus not inactivated, but the GP curves superimposed with those of inactivated complete HIV-1 (Fig. 2B), indicating that AT-2 treatment does not affect HIV-1 membrane lateral structure. Taken together, the results of this series of experiments indicate that Laurdan accurately reports membrane lateral structure and that infectious HIV-1 contains an incomplete membrane, which does not display a phase-transition temperature.

Comparison of Membrane Order, Lipid Composition, and Infectivity of Isogenic HIV-1 Produced from Different Cell Lines—Our previous mass spectrometric analysis of the lipidome of HIV-1 derived from the T-cell line MT-4 showed a strong enrichment in raft lipids (3), and the results reported above revealed a $l_o$ organization for the membrane of HIV-1 particles derived from transfected 293T cells. Subsequently, we directly compared membrane lateral structure, lipid composition, and infectivity of isogenic HIV-1 particles derived from the two different cell lines. For Laurdan staining, HIV-1 particles were produced by transfection of 293T cells or infection of MT-4 cells and were purified, AT-2-inactivated, and labeled using identical protocols.

Emission spectra were recorded for both viruses at various temperatures as described above, and the corresponding GP values were calculated. The two GP curves exhibited a similar shape without detectable $T_m$ (Fig. 3). A significantly higher GP value ($p$ value < 0.0001) was observed for MT-4-derived viruses at all temperatures. The membranes of viruses derived from both MT-4 and 293T cells displayed GP values in the range expected for $l_o$ domains, but with a different degree of membrane order (Fig. 3). Upon treatment with Mβ-CD, HIV-1 particles from both cell lines exhibited a strong decrease in GP values, indicating $l_o$ membrane disruption concomitant with the appearance of an $l_d$ phase and $T_m$ (supplemental Fig. S2).

Lipid extracts from HIV-1 particles and cells were subjected to nano-electrospray ionization tandem mass spectrometry. Total cellular membranes from both cell lines were analyzed in parallel to compare the results with our previous study and to determine potential differences in enrichment or exclusion of specific lipids in virus particles. Comparing the lipidome of isogenic HIV-1 particles from MT-4 and 293T cells revealed a high concentration of raft lipids in both cases (Fig. 4). CHOL concentration was very similar for both viruses amounting to ~45 mol% of total lipids, while SMs ($p$ = 0.050) and phosphatidylserine ($p$ = 0.041) were significantly higher in MT-4-derived viruses. In contrast, PC as well as hexosyl ceramide and ceramide were more concentrated in 293T-derived viruses. The...
hexosyl ceramide species was identified as glucosylerceramide, and no shift in species distribution was observed (data not shown). The concentration of the lipids analyzed in viral and total cell membranes from both cell lines is shown in supplemental Table 1. Comparing the mol% of lipids in HIV-1 particles with the cell line the virus was derived from, an enrichment of presumed raft lipids was again evident (Fig. 5 and supplemental Table 1). HIV-1 particles from both cell lines were clearly enriched in SMs, CHOL, plasmalogen-phosphatidylethanolamine (pl-PE), phosphatidylserine, and hexosyl ceramide at the expense of PC, phosphatidyethanolamine (PE), phosphatidylinositol (PI), and ceramide. A difference in relative enrichment of specific lipids between HIV-1 particles derived from 293T or MT-4 cells was most obvious for SMs. Relative enrichment was ~5-fold for HIV-1 from 293T cells and ~2.5-fold for virus from MT-4 cells (Fig. 5). Despite this higher enrichment factor, 293T-derived viruses still contained a lower mol% of SMs than MT-4-derived virus. In our previous study, we had observed that the unusual SPL DHSM is highly enriched in the membrane of HIV-1 particles from MT-4 cells. In contrast, 293T cells contained no detectable amounts of DHSM, and this was also the case for HIV-1 particles from these cells. CHOL was increased ~3- to 3.5-fold in the viral compared with total cellular membranes in both cases. Furthermore, the ratio of CHOL to total phospholipids was 0.27 and 0.33 for MT-4 and 293T cells, respectively, and 0.78 and 0.89 for the HIV-1 particles produced from these cells.

Isogenic viruses from MT-4 and 293T cells were used for infection of T-cell lines to determine whether the observed differences in lipid composition affect viral infectivity. No difference was observed upon end-point titration of the two viruses in C8166 cells (Fig. 6A). To detect subtle differences, single round infections of MT-4 cells were performed in addition. Under these conditions, HIV-1NL43 from 293T cells exhibited a 2- to 3-fold higher specific infectivity (infectious units per nanogram of viral antigen) than the virus from MT-4 cells (Fig. 6B). The result was different when isogenic HIV-1NL43 was produced from 293T cells transfected with a proviral plasmid rather than by infection with a pseudotyped virus. In this case, relative infectivity of the 293T cell derived virus was 2-fold lower than that of the virus from MT-4 cells.

**Liposomes Composed of HIV-1-derived Lipids Differ in Membrane Lateral Structure from Intact HIV-1—**Cellular as well as viral membranes consist of lipids and proteins. To analyze the relative membrane order in the absence of any proteins, we prepared LUV from lipid extracts of HIV particles (and thus with an identical lipid composition as HIV-1) as well as synthetec LUV with a mixture of commercially available lipids. We subsequently compared their temperature-dependent GP curves with those for authentic HIV-1. Viral lipids were extracted from infectious HIV-1 particles purified from infected MT-4 or transfected 293T cells. The lipid concentration was estimated from the known p24 content of the respective virus preparation assuming 60 lipid molecules per p24 capsid protein as previously determined (3). LUV with a diameter of ~100 nm were prepared from viral lipids at a concentration of ~30 μM; these are referred to as “viral LUV.” In parallel, ~100 nm “synthetic LUV” was prepared from a quaternary lipid mixture consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), DPPC, SM, and CHOL. The relative concentration of each lipid was selected based on the respective lipidomic data: POPC (Tm = 4 °C) was chosen to represent all fluid phospholipids (lα at all measured temperatures) and DPPC (Tm = 41.5 °C) to represent ordered phospholipids (sα and/or lα at all measured temperatures and/or in the presence of CHOL) except for SM. The mol% values of CHOL and SM (corresponding to the sum of SM and DHSM) were derived from the mean value determined for the respective viral lipidsomes. LUV were prepared as described (41) and stained with Laurdan, and emission spectra were recorded at different temperatures. The resulting GP curves for MT-4-derived virus and the respective LUV are shown in Fig. 7A; those for 293T-de-
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**FIGURE 6. Comparison of relative infectivities of isogenic HIV-1 from 293T and MT-4 cells.** Isogenic viruses were produced by infection of MT-4 or 293T cells with VSV-G pseudotyped HIV-1 (MT-4/ VSV or 293T/VSV) or by calcium phosphate transfection of pNL4-3 into 293T cells (293T Tfxn) and used to infect C8166 (A) or MT-4 (B) cells. A, end-point titers in C8166 cells (per nanograms of p24 antigen) were determined after 7 or 8 days (two independent experiments, each in quadruplicate), and results are depicted on a logarithmic scale. B, single-round infectivity in MT-4 cells was determined by fluorescence-activated cell sorting analysis for intracellular p24 capsid antigen 35 h after infection, and results are depicted on a linear scale. The data represent three independent experiments (two for 293T Tfxn) each performed in duplicate and at two or more concentrations of virus input. Error bars represent standard deviation of the mean.

derived virus and LUV are in Fig. 7B. A lower degree of membrane order was observed for either viral or synthetic LUV compared with the authentic virus in both cases, whereas no significant differences in membrane order were observed when comparing viral and synthetic LUV. As expected, given their high CHOL concentration, no transition temperature was observed in either case (44).

**HIV-1 from Different Cell Lines Is Differentially Inactivated by SM-binding Toxins**—As shown above, HIV-1 particles from both cell lines were enriched in SMs, but a significantly higher SMs/PC ratio was observed for MT-4-derived virus than for isogenic virus from 293T cells (Fig. 4). We therefore tested whether SM-binding toxins interfere with HIV-1 infectivity at all and whether a differential effect is observed depending on the SMs/PC ratio of the viral membrane. Inactivation of HIV-1 has previously been shown for several pore-forming toxins (45, 46). Here, we used Eqt-II, a pore-forming toxin from the venom of a sea anemone, *Actinia equinea* (reviewed in Ref. 47). Eqt-II has been shown to directly bind to SM (48) and its pore-forming activity is highly SM-dependent (49, 50). Eqt-II activity was tested in a standard hemolysis assay and shown to be similar as previously reported (supplemental Fig. S3) (51). Treatment of TZM cells with Eqt-II also revealed similar cytotoxicity compared with previously analyzed cell lines (51).

HIV-1 particles produced from either MT-4 or 293T cells were treated with different concentrations of Eqt-II, and their remaining infectivity was monitored 48 h post-infection on TZM reporter cells. MT-4-derived HIV-1 was inhibited in a dose-dependent manner at Eqt-II concentrations >0.2 μg/ml. Infectivity was reduced by almost 80% at 5 μg/ml Eqt-II (Fig. 8A). This reduction in infectivity was not due to cytotoxic effects of Eqt-II on TZM cells, because unbound toxin was inactivated by an excess of serum after incubation with HIV-1, and no effect of Eqt-II on cell viability was observed when the toxin had been preincubated with serum (data not shown). Interestingly, HIV-1 from 293T cells was much more resistant to Eqt-II treatment than MT-4-derived virus, and its infectivity was only decreased by 10–20% even at the highest concentration of Eqt-II (Fig. 8A).

Lysenin, a pore-forming toxin isolated from coelomic fluid of an earthworm, *Eisenia fetida* (52), has also been reported to possess a specific binding capacity to SM (reviewed in Ref. 53). We therefore also analyzed the effect of lysenin on HIV-1 infectivity. Similar to Eqt-II, lysenin blocked HIV-1 from MT-4 cells in a dose-dependent manner, with almost complete loss of infectivity at 5 μg of lysenin per ml (Fig. 8B). Inhibition was less prominent for virus from 293T cells at lower toxin concentrations, whereas both viruses were almost equally inhibited at the higher concentration of lysenin (Fig. 8B). Thus, HIV-1 infectivity and viral entry (data not shown) can be potently blocked by SM-binding agents with preferential inhibition of viruses with higher SM content.

**DISCUSSION**

Many viruses, including HIV-1, are suggested to bud from raft-like microdomains (5, 6), and this was confirmed for HIV-1 by recent mass spectrometric quantitation of its lipidome (3, 4). Raft-like microdomains are presumed to display an ordered lateral membrane structure (l_o phase) due to the high concentration of CHOL, SM, and saturated phosphoglycerolipids (11–13). Here, we applied the phase-sensitive stain Laurdan to probe membrane order of the HIV-1 membrane. Calculating GP values from temperature-dependent emission spectra proved to be a rapid and quantitative method to determine changes in lateral structure of the viral membrane (37) and allowed us to determine producer cell-dependent differences in the HIV-1 membrane. This method may also be useful for a comparative analysis of other viral membranes. Furthermore, a Laurdan-based assay could be applied to screen for lipid-active molecules that interfere with membrane order of pathogenic viruses, which could potentially serve as leads for a new class of antiviral agents.

At room temperature, Laurdan-labeled HIV-1 particles exhibited GP values close to 0.5, and these numbers correlate with the value range established for l_o phase membranes (GP 0.5–0.25) (22, 23). Upon CHOL extraction the GP values were strongly decreased due to the progressive loss of l_o phase, thus validating the assay. Similar GP values as for the HIV-1 membrane were observed when Laurdan was used to probe the
membrane lipid structure of living macrophages, where \( l_o \) domains are particularly enriched on membrane protrusions (filopodia), adhesion points, and cell-cell contacts (26). Furthermore, similar GP-values were also reported for lipid domains at sites of T-cell activation (27). AT-2-inactivated HIV-1 and untreated, non-infectious virus-like particles yielded identical GP patterns indicating that treatment with this compound does not affect viral membrane lateral structure. AT-2 efficiently inactivates HIV-1 by removing Zn\(^{2+}\) from the viral nucleocapsid protein, while preserving antigenicity of surface glycoproteins (36). LUV consisting of lipids extracted from the viral membrane exhibited an altered GP profile compared with the corresponding virus, indicating a higher degree of order in the viral membrane. This difference could be due to the loss of lipid asymmetry in the LUV, where all lipids are distributed equally in both membrane leaflets, whereas PC and SMs are expected to be largely confined to the outer leaflet in the viral membrane. Alternatively or in addition, the lower membrane order in LUV may be affected by the lack of viral proteins (18). It is unlikely that the viral Env glycoproteins have a role in this regard, because the number of Env molecules per virion is low (8–15 trimers) (54–56), and no difference in GP profile was observed for isogenic viruses containing or lacking Env. HIV-1 has been shown to incorporate cellular proteins into the virion membrane, and the inner leaflet of the viral membrane is closely apposed to the hexagonal lattice of the viral matrix protein, which is suggested to stabilize virion architecture. Both of these factors could conceivably contribute to membrane lateral order.

HIV-1 particles containing or lacking viral Env proteins exhibited identical GP profiles. This indicates that incorporation of HIV-1 Env proteins, which appear to sort independently into lipid microdomains, does not influence lateral order of the HIV-1 membrane. This result is consistent with a recent mass spectrometric analysis showing no difference in the lipid composition of HIV-1 particles carrying or lacking Env proteins, and with our own results comparing the lipidomes of HIV-1 with and without Env (data not shown and Ref. 4). Previously, we had shown that the HIV-1 accessory protein Nef

**FIGURE 7. Comparison of GP profiles for HIV-1 and LUV.** Temperature-dependent GP profiles were recorded for HIV-1 from MT-4 cells (A) and from 293T cells (B) as described in Fig. 3 and are displayed as filled circles. GP profiles were also recorded for LUV produced from extracted viral lipids (shaded circles). In addition, LUV were made from a quaternary mixture of 25% POPC, 16% DPPC, 14% SM, and 45% CHOL (A) or 33.5% POPC, 13% DPPC, 8.5% SM, and 45% CHOL (B) to reflect the respective viral lipid compositions, and GP profiles were recorded (open circles). Data are from four independent experiments. Error bars represent standard deviation of the mean.

**FIGURE 8. Effect of SM-binding toxins on HIV-1 infectivity.** Purified HIV-1 produced from either MT-4 cells (●) or 293T cells (○) were treated with Eqt-II (A) or lysenin (B) at the indicated concentration, and their infectivity was determined on TZM cells by measuring luciferase activity 48 h after infection. Untreated samples were set to 100%. Error bars represent standard deviation of the mean.
HIV-1 Laurdan Labeling

HIV-1 exhibits a detectable, but minor influence on the HIV-1 lipidome (57), and an influence of other HIV-1 proteins on viral membrane composition cannot be ruled out at present. It appears likely, however, that the major determinant of membrane composition is the main HIV-1 structural protein Gag. Expression of Gag alone is sufficient to drive formation of virus-like particles in the absence of any other viral components and determination of the lipidome, and the membrane lateral structure of such Gag-only particles will be important to understand the membrane recruitment in HIV-1 budding.

HIV-1 particles pseudotyped with VSV-G also exhibited an identical GP profile as wild-type HIV-1. The efficient pseudotyping of HIV-1 with VSV-G is somewhat surprising, because VSV is believed to bud from non-raft domains of the plasma membrane (58), and there is no evidence for active recruitment of VSV-G by HIV-1 Gag. It appears likely, therefore, that VSV-G in the absence of other viral components distributes throughout the plasma membrane and is thus also present at the HIV-1 budding site. The Laurdan results for the VSV-G pseudotyped particles were again consistent with mass spectrometric analysis of their lipid composition, which showed no difference compared with wild-type HIV-1 (data not shown).

Isogenic viruses from two different cell lines both showed a high mol% of raft lipids and exhibited \( l_o \) phase structure, but to a different degree, as the Laurdan measurements and the phase diagrams revealed. Phase diagrams have been widely studied in synthetic membrane systems, such as liposomes or monolayers, and they are highly relevant for the study of membrane lateral structure. Such phase diagrams can be calculated for different lipid mixtures and provide information regarding their respective order. Phase diagrams formed by the ternary lipid mixture SM, CHOL, and POPC support our results that the HIV-1 membrane is a liquid ordered \( l_o \) membrane (Ref. 59 and references therein). The viral membrane lateral structure was significantly more ordered in HIV-1 particles purified from the T-cell line MT-4 compared with those derived from 293T cells. Lipidomic analyses showed that this difference in membrane order correlated with a higher content of SMs and a higher SM/PC ratio in MT-4-derived viruses, whereas CHOL was similar in MT-4 and 293T virus. It should be noted that the relative enrichment of SMs in HIV-1 particles compared with whole cell membranes was even more pronounced in virus from 293T cells compared with virus from MT-4 cells, while the mol% of SMs was lower. It is difficult to evaluate this difference, however, because no comparison with plasma membrane (where the virus buds) could be made, and the contribution of plasma membrane to total cell membrane may not be equal in different cell types. Chan et al. recently compared the lipid composition of HIV-1 and the plasma membrane of producer cells (4). These authors reported no significant difference in most lipids, including SM between viral membrane and plasma membrane, while CHOL, ceramide, glycosphingolipids, and inositol 1,4,5-bisphosphate were enriched in the viral membrane. Viruses and cell membranes were derived from the H9 T-cell line and from primary human macrophages in this case, however, and our results therefore cannot be directly compared with this study. The different lipid composition of isogenic HIV-1 from MT-4 and 293T cells did not detectably alter viral infectivity when end-point titers for viruses from both cell lines were determined in parallel. Applying a more sensitive single-round assay, virus from 293T cells exhibited a slightly (2- to 3-fold) better infectivity than isogenic HIV-1 from MT-4 cells, when both viruses were produced the same way. This increased infectivity was not maintained, however, when the production method was altered. Taken together these results indicate that the observed differences in lipid composition do not cause significant changes in infectivity at least in the cell lines used. Conceivably, they may correspond to more subtle effects on viral stability and more detailed analyses of viral decay kinetics will be required to address this issue.

Lowering of CHOL (6) or SPL (3, 60, 61) in the producer cell as well as extraction of CHOL (5–7) from HIV-1 particles has been shown to interfere with viral infectivity in previous studies, and this effect appears to be dependent on the target cell. Here, we show that SM-binding toxins also block HIV-1 infectivity in an SM concentration-dependent manner. Experiments with SM-binding toxins were originally initiated to introduce pores into the viral membrane and thereby gain access to inner virion structures. Detailed studies, including protease sensitivity of inner core proteins and stain penetration in negative stain electron microscopy, indicated, however, that pores were not consistently introduced into the viral membrane despite complete inactivation of viral infectivity (data not shown). It appears likely, therefore, that inactivation is primarily caused by toxin binding to the viral membrane (which was observed by EM analysis, data not shown). More efficient inactivation of MT-4-derived virus may then be most easily explained by its higher SMs concentration compared with 293T cell-derived virus and the consequent higher number of binding sites for the toxin. While pore-forming toxins certainly cannot serve as drug candidates, the assay reported here may facilitate testing of other lipophilic compounds that bind and alter HIV-1 membrane structure. Success of an antiviral approach targeting the viral membrane structure clearly depends on achieving sufficient specificity for the viral compared with cellular membranes. Such approaches may be particularly interesting for the development of mucosal microbicidal inactivating the virus.

Acknowledgments—We are grateful to A. Habermann and J. Krijnse-Locker for EM analysis, I. Allespach and C. Goffinet for fluorescence-activated cell sorting support, T. Sachsheimer and M. Haag for mass spectrometry support, V. Bosch for providing plasmid ΔEnvpNL4-3, I. Leibrecht for technical assistance, and I. Morales and F. X. Contreras for critical reading of the manuscript.

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