

Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity

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Objective: HIV-1 particles are enriched with cholesterol; however, the significance of this cholesterol enrichment is unknown. This study examines the structural and functional roles of cholesterol in HIV-1 replication.

Methods: Using methyl- β -cyclodextrin (CD) to remove cholesterol from the HIV-1 envelope, buoyant density and infectivity of the cholesterol-deficient HIV-1 particles were compared with the untreated control. The specificity and requirement of cholesterol as an HIV-1-associated lipid were investigated by replenishing cholesterol-deficient HIV-1 with cholesterol, cholestenone (a cholesterol structural analogue) or sphingomyelin (a structurally unrelated yet virion-associated lipid).

Results: CD-mediated removal of virion cholesterol increased the buoyant density of virion particles and reduced HIV-1 infectivity. *Trans*-supplementation of exogenous cholesterol rescued the defects associated with CD-induced cholesterol depletion in HIV-1. However, the restoration of viral infectivity could not be achieved by *trans*-supplementation of either cholestenone or sphingomyelin.

Conclusion: This study provides the first direct evidence that HIV-1-associated cholesterol is important for the maintenance of virion structure and infectivity. While the buoyant density of cholesterol-defective HIV-1 can be restored by a cholesterol structural analogue, cholestenone, the requirement for cholesterol is essential for HIV-1 infectivity.

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AIDS 2002, **16**:2253–2261

Keywords: envelope proteins, lipid, molecular biology, retrovirus, viral infections, virus proteins

Introduction

Enveloped viruses, including HIV-1, incorporate a lipid bilayer from the host cell membrane to form their virion envelope structure [1–5]. A number of enveloped viruses, including influenza, measles, Semliki Forest virus, Sendai virus, sindbis virus and vesicular stomatitis virus, contain high levels of cholesterol in their virion envelopes. These viruses rely on cholesterol in their viral replication cycles for the assembly of cholesterol-rich lipid rafts and for entering the host cell through cholesterol-rich microdomains [6–14]. The requirement for cholesterol or lipid rafts is not exclusive to viruses, as cholesterol is required by mycobac-

teria and other bacterial pathogens (for reviews see [15,16]).

Analysis of the lipid content of Rous sarcoma virus provided the first indication that cholesterol is important for retroviral replication [17]. Subsequent work has shown that HIV-1 infection alters the cholesterol:phospholipid ratio in the plasma membrane of host cells [18,19]. Accumulating data suggest that HIV-1 relies on cholesterol at many stages of its replication cycle, including entry, assembly and release (for review see [20]). For example, the HIV-1 receptor CD4 and coreceptors CXCR4/CCR5 are found within lipid rafts [21–24], and these cholesterol-rich membrane

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Received: 25 January 2002; revised: 2 May 2002; accepted: 29 July 2002.

microdomains undergo rearrangement upon gp120-CD4 engagement, presumably to facilitate coreceptor interaction and subsequently permit HIV-1 infection [25]. This fusion process can be suppressed by depletion of membrane-associated cholesterol, possibly by affecting downstream signalling events upon binding of viral coreceptors [26]. The organization of lipids in the lipid rafts of target cells and the mature HIV-1 envelope lipid bilayer may in part regulate the fusion process. HIV-1 infection relies on multiple intermolecular interactions that include contact between HIV-1 gp120 and surface glycosphingolipids, such as galactosylceramide [27], Gb3 and GM3 [28]. Interestingly, these glycosphingolipids are critical for the lateral arrangement of lipid rafts [29].

Cholesterol-rich lipid rafts serve as the assembly and release sites for HIV-1 [30], and formation of these HIV-1-associated lipid rafts may be driven by the localization of HIV-1 Gag protein to the plasma membrane [31,32]. The palmitoylation of HIV-1 envelope in the Golgi is critical for the association of Env with lipid rafts and subsequent packaging of Env into the virion [33]. The HIV-1 envelope is substantially enriched in cholesterol in comparison with the host cell membrane [19]. The cholesterol content of the lipid envelope represents 15% by weight of HIV-1, while viral proteins and genomic RNA provide only 50% and 2% of its weight, respectively [34]. It is clear that membrane-associated cholesterol in the target cells is critical for HIV-1 infection and syncytium formation [26]; however, the functional significance of cholesterol enrichment in mature HIV-1 remains speculative.

The functional role of cholesterol in mature HIV-1 has been investigated by altering the cholesterol composition of infectious HIV-1 via treatment with a specific cholesterol-binding agent, methyl- β -cyclodextrin (CD).

Methods

Virus production

Wild-type HIV-1 particles were produced by calcium phosphate transfection of proviral DNA into 293T cells, as previously described [35]. Cells were transfected with 5 μ g HIV-1 proviral DNA (HXB2-BH10) per dish [36]. HIV-1 particles containing the luciferase reporter gene in place of *nef* were produced by cotransfecting 10 μ g HIV-1 luciferase reporter proviral DNA pNL4-3.Luc.R-E- and 5 μ g HIV-1 envelope and accessory proteins expression vector pNLA1 per dish. pNL4-3.Luc.R-E- is a *nef*-defective HIV-1 proviral DNA that contains two frameshift mutations to suppress functional expression of Env and Vpr [37]. The pNL4-3.Luc.R-E- clone was obtained through the AIDS Research and Reference Reagent Program,

from Dr Nathaniel Landau. pNLA1 is an HIV-1 Env and accessory proteins expression vector (a kind gift from Dr Damian Purcell, University of Melbourne) that expresses all HIV-1 proteins with the exception of Gag and GagProPol. Cotransfection of pNL4-3.Luc.R-E- and pNLA1 into 293T cells provides all the viral proteins for the production of infectious HIV-1. The lack of functional *env*, *vpr* and *nef* reading frames in the packaged pNL4-3.Luc.R-E- genome, however, means that a second round of viral replication cannot be supported. Successful single-round infection by pNL4-3.Luc.R-E-/pNLA1 virion particles was detected by expression of the luciferase reporter gene. Viral particles were purified from the supernatant and concentrated by ultracentrifugation through 20% sucrose, using an L-90 ultracentrifuge (SW41 rotor; Beckman, Fullerton, California, USA) at 87 000 $\times g$ for 1 h at 4°C. Virus pellets were eluted in phosphate-buffered saline (PBS).

Methyl- β -cyclodextrin and cholesterol treatment of HIV-1

A known amount of HIV-1 [corresponding to 1.3 μ g p24 capsid protein (CA)] was treated with up to 1 mmol/l CD (Sigma; St Louis, Missouri, USA) in PBS at 37°C for 1 h to remove cholesterol from the mature HIV-1 lipid envelope. After 1 h of 0.5 mmol/l CD treatment of concentrated virus, 0.025, 0.25 or 0.5 mmol/l exogenous cholesterol (Sigma) was added to the CD-treated virus for 1 h at 37°C to replenish the virion-associated cholesterol. These exogenous cholesterol concentrations represented 1-, 10- and 20-fold virion-associated cholesterol per virion particle [34]. Cholestenone (5-cholesten-3-one; Sigma) is a structural analogue of cholesterol that has a 3-C ketone group in place of the 3 β -hydroxyl group of cholesterol (see insert, Fig. 3b, below), which limits the hydrogen-bonding capacity of the molecule compared with that of cholesterol. Cholestenone was supplied to CD-treated HIV-1 in a similar manner to the cholesterol replenishment described above. After 1 h of 0.5 mmol/l CD treatment, 0.05 or 0.25 mmol/l exogenous cholestenone was mixed with CD-treated virus for 1 h at 37°C. These concentrations of exogenous cholestenone represent 2- and 10-fold higher virion-associated cholesterol per virion particle. A HIV-1-associated yet structurally unrelated lipid, sphingomyelin, was used to treat the cholesterol-depleted viral particles. After 0.5 mmol/l CD treatment of concentrated virus, 0.05 or 0.25 mmol/l exogenous sphingomyelin was added to the CD-treated virus for 1 h at 37°C. These concentrations of exogenous sphingomyelin represent 2- and 10-fold higher virion-associated cholesterol per virion particle. All exogenous lipids were firstly dissolved in ethanol and subsequently introduced to CD-treated HIV-1. The same amount of ethanol was added to the mock- and CD-treated HIV-1 without lipid replenishment.

OptiPrep and sucrose gradient analysis

OptiPrep velocity gradient ultracentrifugation was used to separate virion particles from soluble viral proteins. OptiPrep [60% (w/v) iodixanol; Sigma] gradients were prepared in PBS in 1.2% increments from 6 to 18% iodixanol as previously described [38]. Briefly, CD-treated virions were layered onto the gradient and centrifuged for 45 min at $110\,000 \times g$ (SW41 rotor; L-90 Ultracentrifuge; Beckman). The specific density of CD-treated HIV-1 was determined by sucrose density gradients prepared in PBS in 2.5% increments ranging from 30 to 55% sucrose (Gibco/BRL, Gaithersburg, Maryland, USA). This range of sucrose gradients yields a density of 1.147–1.149 g/ml for wild-type HIV-1. The same stock of HIV-1 has a density of 1.16 g/ml in a 20–60% sucrose gradient. CD-treated HIV-1 preparations were layered onto the gradient and centrifuged for 16 h at $87\,000 \times g$ (SW41 rotor; L-90 Ultracentrifuge; Beckman). Fractions were collected from the top. Reverse transcriptase (RT) activity and immunodetection of HIV-1 p24 CA were used to determine the location of virion particles in the gradient fractions [38]. The precise density of each sucrose gradient fraction was determined using a refractometer (Carl Zeiss, Germany, kindly provided by Dr Chris Birch, Victorian Infectious Diseases Reference Laboratory) and significance was assessed using the Students t-test (paired, two-tailed).

Immunoblotting

Protein extracts derived from gradient fractions were resuspended in $1 \times$ loading buffer [20 mmol/l Tris-HCl pH 6.8, 0.3% β -mercaptoethanol, 2% sodium dodecylsulphate, 7% glycerol and 0.1% bromophenol blue] and resolved by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis. Viral proteins were identified using pooled sera from HIV-1-seropositive patients, anti-gp41 monoclonal antibody [gp41 antibody was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, US National Institutes of Health (NIH): Chessie 8 from Dr George Lewis [39]] or anti-gp120 monoclonal antibody [gp120 antibody was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: monoclonal antibody to gp120 (No. 902) from Dr Bruce Chesebro [40,41]] and followed by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase. Specific proteins were visualized by enhanced chemiluminescence according to manufacturer's instructions (Amersham Pharmacia Biotech; Amersham, England).

Reverse transcriptase assay

Samples of 10 μ l of each gradient fraction sample were mixed with 10 μ l 0.3% Nonidet P-40, followed by addition of 40 μ l RT reaction cocktail containing the template primer 5 μ g/ml poly(rA)-(dT)₁₅ (Amersham Pharmacia Biotech) in 50 mmol/l Tris-HCl pH 7.8,

2 mmol/l dithiothreitol, 5 mmol/l MgCl₂, 7.5 mmol/l KCl, 0.5 μ Ci [α ³³P]-dTTP. Following a 3 h incubation at 37°C, 8 μ l of the reaction mixture was spotted onto DEAE ion-exchange paper (Whatman, Maidstone, UK) and washed six times in 300 mmol/l NaCl plus 30 mmol/l sodium citrate to remove unincorporated [α ³³P]-dTTP. RT activity was determined by the level of [α ³³P]-dTTP bound to the DEAE filter using a 1450 Microbeta Plus liquid scintillation counter (Wallac, Finland).

Infectivity assay

A single round of HIV-1 replication was used to assess the infectivity of CD-treated virus in a MT2 cell line [37]. MT2 cells were maintained in RF10 [RPMI-1640 (Gibco/BRL) containing 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine and 24 μ g/ml gentamicin]. HIV-1, with a luciferase reporter gene in place of the *nef* gene coding sequence within the RNA genome, was treated with 0.5 mmol/l CD. The untreated and CD-treated HIV-1 was subsequently purified by sucrose equilibrium gradients to remove CD and soluble HIV-1 proteins. These viruses were used to infect the MT2 cells with an equivalent input of p24 CA protein. HIV-1 Nef, Vpr and Env were supplied by pNLA1 during cotransfection for the production of one-round replicating, luciferase reporter viruses. Prior to infection, the pNL4-3.Luc.R-E-/pNLA1 virion particles were treated with 0–0.5 mmol/l CD and purified using a 30–55% sucrose density gradient (as described previously). In some cases the CD-treated HIV-1 was replenished with exogenously supplied cholesterol, cholestenone or sphingomyelin. The peak virus-containing fractions were isolated and virus particles were purified and concentrated. Equivalent amounts (as quantified by p24 assay) of untreated and modified HIV-1, were used to infect 1×10^6 MT2 cells (in RPMI-1640, 2 mmol/l L-glutamine and 24 μ g/ml gentamicin) for 1 h at 37°C in the absence of cholesterol or serum. Residual virus was removed by PBS wash and cells were resuspended in 1 ml RF10 at 37°C for 48 h. The success of infection was determined by the level of luciferase activity in the MT2 cells by the Luciferase Assay System (Promega, Madison, WI, USA). The impact of CD treatment and virion lipid modification on HIV-1 infectivity are represented as a percentage (mean \pm SE of triplicate samples) of untreated controls. The significance of the result was assessed using the Students t-test (paired, two-tailed).

Results

The effect of methyl- β -cyclodextrin treatment on the stability and buoyant density of HIV-1

The velocity gradient migration profile of untreated HIV-1 particles displayed a peak of viral proteins, represented by comigration of RT activity and CA, at

fraction 10 (Fig. 1a). Upon treatment of HIV-1 with CD (0, 0.25, 0.5 and 1.0 mmol/l) to remove virus-associated cholesterol, there was a subtle, dose-dependent increase in the migration rate of the viral particles. A corresponding increase in the amount of viral proteins detected as soluble p24 CA and RT proteins in fractions 1 and 2 was also observed. To investigate whether the different mobilities of CD-treated and untreated HIV-1 in the velocity gradient were asso-

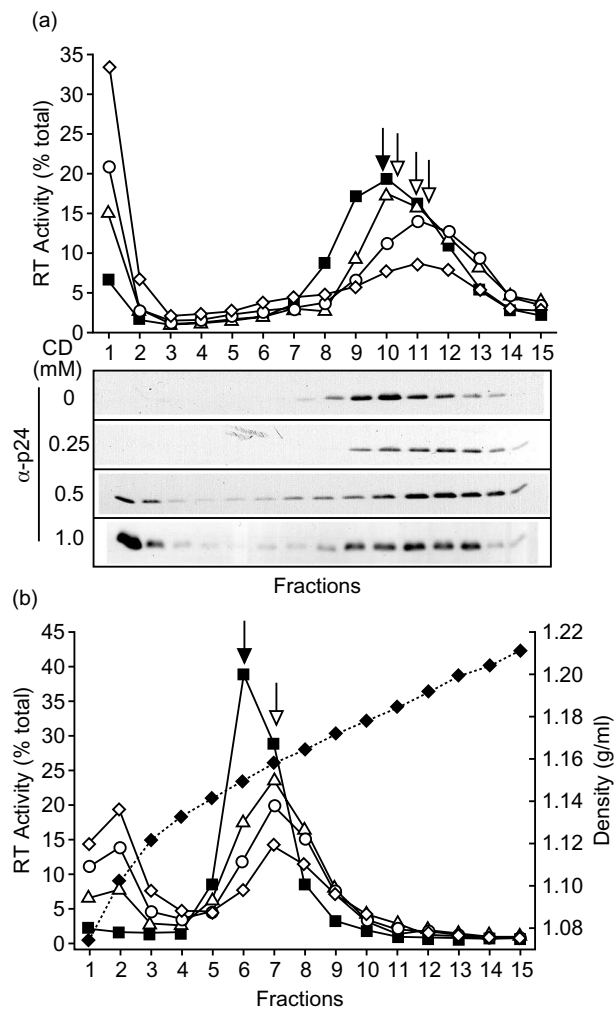


Fig. 1. Methyl- β -cyclodextrin (CD) treatment of HIV-1. (a) Reverse transcriptase (RT) activity and p24 capsid protein detected in OptiPrep velocity gradient, which separates virion particles from soluble viral proteins (representative of 10 experiments). (b) The presence of virion particles, as determined by viral RT activity, in sucrose density equilibrium gradients. RT activity (solid line) was detected in each fraction, for which duplicate data points of percentage total RT activity varied by $< 3\%$ (SE). Corresponding densities (dashed line) of each fraction were determined by refractive index measurements, representative of five experiments, where duplicate measurements varied by 0.001 g/ml (SE). Untreated HIV-1 (■); HIV-1 treated with 0.25 (Δ), 0.5 (○) and 1 mmol/l (◇) CD.

ciated with alterations in virion density, CD-treated HIV-1 was examined using sucrose density equilibrium gradient analysis. Untreated HIV-1 particles sediment at approximately 1.15 g/ml, as detected by a peak of RT activity (Fig. 1b; fraction 6). Upon treatment of HIV-1 with CD (0, 0.25, 0.5 and 1.0 mmol/l), a shift to a higher buoyant density viral particle (~ 1.16 g/ml) was consistently observed (Fig. 1b). In contrast to the mobility of HIV-1 treated with 0.25, 0.5 and 1.0 mmol/l CD (Fig. 1a), a dose-dependent increase in particle density was not observed among these CD-treated HIV-1 (Fig. 1b). The cholesterol-deficient CD-treated virus existed at a defined density before it destabilized to produce dose-dependent soluble RT proteins at the top of the gradient. The precise densities of HIV-1 were determined by the refractive index of sucrose in each gradient fraction using a refractometer. Cholesterol-depleted HIV-1 particles consistently had a density of 1.158 ± 0.001 g/ml, while untreated HIV-1 had a density of 1.148 ± 0.001 g/ml ($P = 0.001$; $n = 4$).

The effect of cholesterol depletion on HIV-1 infectivity

The functional significance of cholesterol depletion on HIV-1 replication was examined in the single-round infectivity assay [37]. Treatment of HIV-1 with 0.5 mmol/l CD was associated with 40–60% removal of virion-associated cholesterol (data not shown), which corresponds to an approximate 50% reduction in viral infectivity. This was statistically significant ($P = 0.003$; $n = 4$) compared with the untreated control (Fig. 2b).

The reversibility of cholesterol depletion

To assess whether the effect of CD on HIV-1 is permanent or reversible, exogenous cholesterol was used to replenish the reduced level of virion-associated cholesterol in CD-treated HIV-1. It has been estimated that the cholesterol content of HIV-1 is approximately 2.5-fold, by mass, of the viral p24 [34]. Accordingly, 1.3 μ g p24 equivalent of CD-treated HIV-1 was incubated with 0.025, 0.25 and 0.5 mmol/l cholesterol in 300 μ l reaction mix, which represented 1:1, 1:10 and 1:20 exogenous cholesterol:virion-associated cholesterol, respectively (Fig. 2). Exogenously supplied cholesterol was able to restore the effect of CD treatment on HIV-1 infectivity (Fig. 2b), in that the level of infection was not significantly different from that of the untreated control ($P = 0.46$; $n = 4$). Furthermore, the profile of the sucrose density equilibrium gradient clearly indicated that replenishment of CD-treated HIV-1 with 1:1 exogenous cholesterol:virion-associated cholesterol rescued the density of the viral particle back to that of the untreated control (Fig. 2a). Interestingly, virion-associated cholesterol is not readily exchanged with exogenous cholesterol in the absence of CD treatment (data not shown). Furthermore, intact HIV-1 incubated

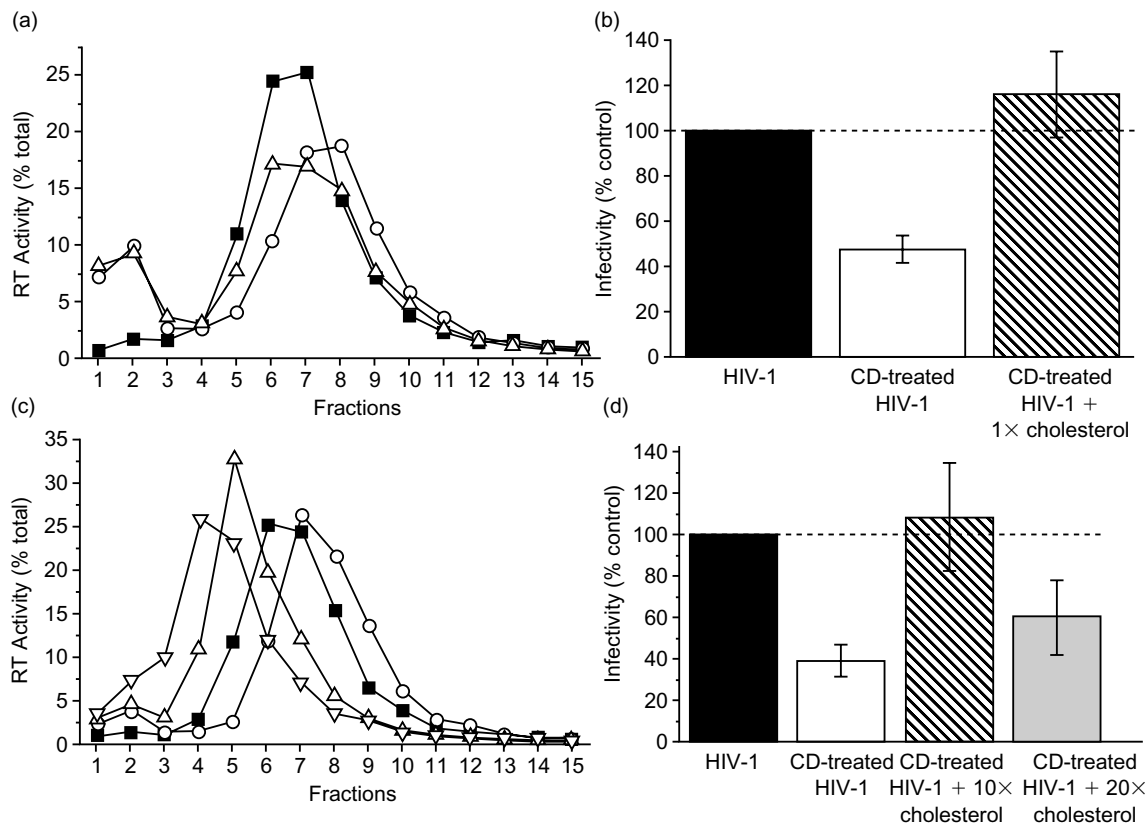


Fig. 2. Density gradient profile and viral infectivity analysis of methyl- β -cyclodextrin (CD)-treated HIV-1 and CD-treated HIV-1 incubated with exogenous cholesterol. HIV-1 containing the luciferase reporter gene was mock-treated, CD-treated (0.5 mmol/l) or CD-treated (0.5 mmol/l) and replenished with exogenous cholesterol. Treated and untreated HIV-1 were normalized for p24 capsid protein content and used to infect MT2 cells. The level of luciferase activity was used as an indicator of infectivity, which was reported as a percentage (mean \pm SE of triplicate samples) of the untreated control. Duplicate percentage total reverse transcriptase (RT) activity data points varied by $< 3\%$ (SE) (a,c). (a) Density gradient profiles of RT activity for untreated HIV-1 (■), CD-treated HIV-1 (○) and CD treated HIV-1 that has subsequently been replenished with 10:1 (Δ) exogenous cholesterol:virus-associated cholesterol. (b) Infectivity levels of the HIV-1 preparations in (a). (c) Density gradient profiles of RT activity for untreated HIV-1 (■), CD-treated HIV-1 (○) and CD treated HIV-1 that has subsequently been replenished with 10:1 (Δ) or 20:1 (∇) exogenous cholesterol:virus-associated cholesterol. (d) Infectivity levels of the HIV-1 preparations in (c). Data are representative of three experiments.

with exogenous cholesterol displayed no detectable effect on viral infectivity and density profile (data not shown).

The effect of replenishment of cholesterol in depleted HIV-1 on virion particle density and viral infectivity

To examine whether CD-treated HIV-1 are able to accommodate excess cholesterol and whether this affects virion structure and function, the cholesterol-depleted viral particles were supplied with 10:1 and 20:1 exogenous cholesterol:virus-associated cholesterol (Fig. 2c,d). Supplementation of CD-treated HIV-1 with 10- and 20-fold excess exogenous cholesterol shifted the density profile of the viral particles to buoyant densities lower than the untreated control (Fig. 2c). These two types of virus-like particle (formed by 10- and 20-fold excess cholesterol), however, consistently had a marked difference ($P = 0.01$; $n = 4$)

in viral infectivity (Fig. 2d), implying that there is an upper limit of virion-associated cholesterol in an infectious HIV-1 particle. The infectivity of particles formed with 10- and 20-fold excess cholesterol compared with the untreated control was not significantly different ($P = 0.77$, $n = 4$ and $P = 0.12$, $n = 4$, respectively). In contrast, incubation of 10- and 20-fold excess cholesterol with non-CD-treated HIV-1 did not change the buoyant density or infectivity of HIV-1 (data not shown).

The effect of added cholesterol to depleted HIV-1 particles

To examine the specificity of cholesterol in the HIV-1 envelope, cholesterol was used in place of cholesterol in an attempt to restore the effects of CD on HIV-1. Incubation with 2:1 or 10:1 exogenous cholesterol:virus-associated cholesterol was required to shift the density profile of CD-treated HIV-1 back within the

range of the untreated HIV-1 profile (Fig. 3a). In comparison with cholesterol replenishment of CD-treated HIV-1, 2- to 10-fold cholestenone was required to cause a similar density profile reversion (Fig. 2a and Fig. 3a) while 1-fold exogenous cholestenone was without effect (data not shown). This implies that the different oxidative states of cholesterol and cholestenone at the 3-C position may reduce the binding affinity to the HIV-1 envelope of the latter, and a higher concentration of cholestenone is required to compensate for the reduction in binding kinetics. Infectivity studies revealed that viral particles replenished with cholestenone were less infectious ($P < 0.01$; $n = 3$) than untreated wild-type HIV-1 (Fig. 3b). Moreover, cholestenone association with the CD-treated particles further reduced the infectivity to $\sim 10\%$ of the untreated control (Fig. 3b).

The effect of sphingomyelin on depleted HIV-1

Sphingomyelin is a major component of lipid rafts [42]

and is also incorporated into the HIV-1 lipid envelope during budding of the virus from an infected cell [34]. It has been shown that upregulation of sphingomyelin production can substitute for other lipids in plasma membrane lipid-raft structures [43]. To examine whether sphingomyelin can be substituted for HIV-1-associated cholesterol, 2:1 and 10:1 exogenous sphingomyelin:virus-associated cholesterol was incubated with CD-treated HIV-1. Sphingomyelin did not reverse the increase in virus particle density caused by CD treatment (Fig. 3c). In addition, a further dose-dependent increase in buoyant density was observed in CD-treated HIV-1 incubated with exogenous sphingomyelin (Fig. 3c). This may be a result, in part, of the difference in molecular structure of sphingomyelin and cholesterol. Unlike cholesterol or cholestenone, the larger structure of sphingomyelin may not favour incorporation into a cholesterol-depleted virus structure. Unexpectedly, the infectivity of CD-treated HIV-1 replenished with sphingomyelin was also reduced to less than $\sim 10\%$ of

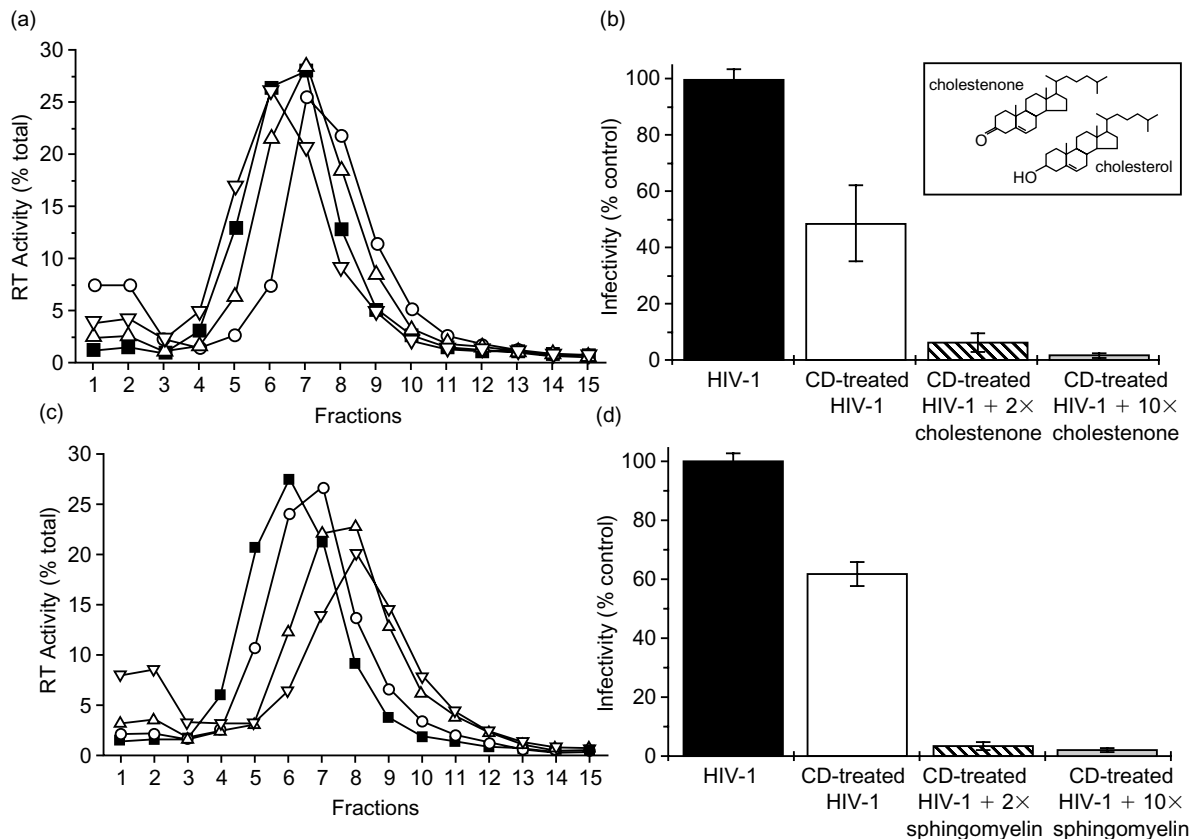


Fig. 3. Density gradient profile and viral infectivity analysis of methyl- β -cyclodextrin (CD)-treated HIV-1 incubated with exogenous cholestenone and sphingomyelin. Cholestenone and cholesterol structures are shown in the inset. All CD treatment is at 0.5 mmol/l. (a) Density gradient profile of CD-treated HIV-1 and subsequently replenished with 2:1 (Δ) and 10:1 (∇) exogenous cholestenone:virus-associated cholesterol compared with untreated HIV-1 (\blacksquare) and CD-treated HIV-1 (\circ). (b) Infectivity of the HIV-1 preparations in (a). (c) Density gradient profile of CD-treated HIV-1 subsequently replenished with 2:1 (Δ) and 10:1 (∇) exogenous sphingomyelin:virus-associated cholesterol compared with untreated HIV-1 (\blacksquare) and CD-treated HIV-1 (\circ). (d) Infectivity of the HIV-1 preparations in (c). Infectivity is represented as a percentage (mean \pm SE of triplicate samples) of the untreated HIV-1 control. Duplicate percentage total reverse transcriptase (RT) activity data points varied by $< 3\%$ (SE). Data are representative of at least three sets of experiments.

the untreated control ($P < 0.01$; $n = 3$) (Fig. 3d). The opposing effects of cholestenone and sphingomyelin on the buoyant density of CD-treated HIV-1 imply that these two compounds are likely to act through distinct mechanisms to suppress viral infectivity.

The effect of methyl- β -cyclodextrin and lipid replenishment on envelope proteins

HIV-1 particles lacking viral envelope proteins are unable to infect target cells. The additional reduction in viral infectivity of CD-treated HIV-1 upon incubation with cholestenone and sphingomyelin may in part be caused by interference with the anchoring of the envelope protein onto the virion lipid bilayer. Immunoblotting for gp41 and gp120 envelope proteins in untreated, CD-treated HIV-1 or CD-treated HIV-1 subsequently replenished with lipid showed wild-type levels of HIV-1 gp41 and gp120 (Fig. 4). Our data suggest that the reduction in infectivity associated with cholesterol depletion or lipid replenishment of CD-treated HIV-1 cannot be attributed to a reduction in HIV-1 envelope proteins on the virion surface.

Discussion

A number of recent reports have demonstrated the importance of cholesterol and lipid rafts in HIV-1 replication; however, no study has examined the contribution of virion-associated lipid in HIV-1 replication. Our studies provide the first direct evidence that HIV-1-associated cholesterol is critical for viral structure and infectivity.

The cholesterol:phospholipid ratio of HIV-1 is significantly higher than that of the host cell plasma membrane [18,19]. It has been reported that HIV-1 buds from lipid rafts [30]; these lipid rafts are specialized membrane microdomains that have distinctively high levels of cholesterol, sphingolipid and glycolipid [42]. As HIV-1 gains its cholesterol content during budding through lipid rafts, it is conceivable that the packaging arrangement of viral-associated lipids are in a conformation that mimics cellular lipid raft structures. Interestingly, influenza virus has been shown to incorporate lipid raft-like structures into the virus envelope during budding [9] and a number of lipid raft-associated proteins are also found in HIV-1 [30]. These highly ordered structures may provide a rigid, envelope structure to protect the virion core from the extracellular milieu.

Lipid rafts are highly ordered structures that are enriched with saturated lipids (such as sphingomyelin). It is thought that cholesterol intercalates between the lipid acyl chains to fill the voids between the saturated acyl chains of sphingolipids [44]. The 3β -hydroxyl

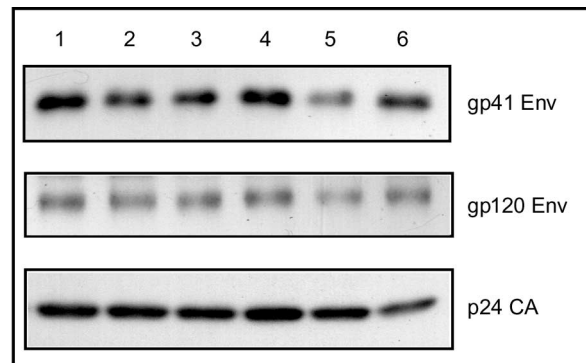


Fig. 4. Immunoblot analysis of HIV-1 gp41 and gp120 envelope proteins and p24 capsid protein (CA) present on viral particles after methyl- β -cyclodextrin (CD; 0.5 mmol/l) treatment and subsequent replenishment with exogenous cholesterol, cholestenone or sphingomyelin. Concentrated HIV-1 particles were treated and then isolated from peak reverse transcriptase activity fractions in sucrose equilibrium density gradients as described in the Methods. Contamination of microvesicles in the virus suspension was removed by an additional Optiprep gradient purification. Lane 1, untreated HIV-1 (control); lane 2, CD-treated HIV-1; lane 3, CD-treated HIV-1 replenished with 10:1 exogenous cholesterol:virus-associated cholesterol; lane 4, CD-treated HIV-1 replenished with 20:1 exogenous cholesterol:virus-associated cholesterol; lane 5, CD-treated HIV-1 replenished with 2:1 exogenous cholestenone:virus-associated cholesterol; lane 6, CD-treated HIV-1 replenished with 2:1 exogenous sphingomyelin:virus-associated cholesterol.

group of cholesterol is of particular importance as it acts as both a hydrogen bond-accepting and hydrogen bond-donating group to stabilize the amide bond within the polar head group of sphingolipids [44]. This interaction also enhances the tight packaging of sphingolipids by restricting cholesterol to a defined depth within the lipid bilayer [45–47]. *In vitro* analysis has shown that the 3β -hydroxyl group of cholesterol induces the formation of lipid raft-like structures, whereas the 3-C ketone group in cholestenone prohibits lipid domain formation [48]. These findings are consistent with our observation that a modestly higher concentration of exogenous cholestenone than cholesterol was required to rescue the HIV-1 density profile, possibly reflecting a less favourable binding affinity between cholestenone and the virion lipid envelope compared with that of cholesterol and the virion envelope. If the function of cholesterol in the virion envelope is merely space filling, one would anticipate that the replenishment of CD-treated HIV-1 with cholestenone would restore viral infectivity. The additional suppression of viral infectivity in cholestenone-replenished CD-treated HIV-1 supports our notion that the packaging arrangement of cholesterol in the virion envelope is also important for viral replication, such that lipid raft-like structures within the virion

envelope may be required for the release of the virion core into target cells during viral entry.

Xu and London [48] have reported that the cholesterol ring structures are also important for the formation of lipid raft-like domains *in vitro*. For example, both 5 α -cholestan-3 β -ol (dihydrocholesterol) and 5 β -cholestan-3 β -ol (coprostanol) consist of a 3 β -hydroxyl group and only differ by the chirality in the fifth carbon. These two cholesterol analogues, however, show opposing effects on the formation of lipid raft-like structures *in vitro* [48] with dihydrocholesterol promoting lipid raft formation and coprostanol disrupting it. If virion-associated cholesterol acts to stabilize lipid raft-like structures in HIV-1, one would then expect CD-treated HIV-1 replenished with dihydrocholesterol to have wild-type infectivity, while coprostanol-replenished CD-treated HIV-1 would be non-infectious. Treating mature HIV-1 with CD, we have developed a novel system to replace virion-associated cholesterol with a variety of cholesterol analogues. Analysis of mature HIV-1 with different cholesterol analogues may help to review the requirement for a lipid-raft-like structure in the HIV-1 envelope.

It has been shown that sphingomyelin and glycosphingolipids are found in similar quantities in lipid rafts [49]. Interestingly, a mutant melanoma cell line (GM-95) that is defective in glycosphingolipid synthesis has a heightened level of sphingomyelin synthesis to compensate for the lack of glycosphingolipids in the formation of lipid raft-like structures [43]. In contrast to sphingomyelin's ability to substitute for other sphingolipids in lipid rafts, replenishment of sphingomyelin in CD-treated, cholesterol-defective HIV-1 restored neither virion buoyant density nor viral infectivity. This may, in part, result from the large structural difference between sphingomyelin and cholesterol. However, the unexpected suppression of infectivity of sphingomyelin-replenished CD-treated HIV-1 may suggest a dynamic relationship between cholesterol and sphingomyelin in the virion envelope of HIV-1. For example, the affinity between sphingomyelin and cholesterol is one of the driving forces for lipid raft formation. Excess sphingomyelin in the virion particle may further disrupt the positioning of cholesterol within the virion membrane, which may lead to further suppression of viral infectivity. Similar to virion-associated cholesterol, it is not known whether sphingomyelin is functionally required for viral replication. Interestingly, the cleavage of sphingomyelin has been shown to be critical for sindbis virus-induced apoptosis during viral entry [50]. Analysis of HIV-1 treated with sphingomyelinase may help to elucidate this lipid's role in the replication of HIV-1.

Our work provides the first direct evidence that enrichment of cholesterol in the mature HIV-1 is

critical for the maintenance of virion structure and function. This work also provides the first glimpse of the arrangement of lipids in the virion envelope. The requirement for cholesterol and sphingomyelin in the lipid envelope of HIV-1 and its particular lipid-packing arrangement may hold novel avenues for anti-retroviral therapy development.

Acknowledgements

We thank John Mills and Melissa Hill for critical review of the manuscript and Andy Pountourios (St Vincent's Medical Research Institute) for purifying the HIV-1 Env antibodies (NIH AIDS Reagents Program). We also thank Chris Birch (Victorian Infectious Diseases Reference Laboratory) for the use of the refractometer.

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