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Sphingolipids, cholesterol, and HIV-1: A paradigm in viral fusion

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Abstract Our previous studies show that the depletion of cholesterol or sphingolipids (raft-associated lipids) from receptor-bearing adherent cell lines blocks HIV-1 entry and HIV-1 Env-mediated membrane fusion. Here we have evaluated the mechanism(s) by which these lipids contribute to the HIV-1 Env-mediated membrane fusion. We report the following: (1) GSL depletion from a suspension T lymphocyte cell line (Sup-T1) reduced subsequent fusion with HIV-1IIIB-expressing cells by 70%. (2) Cholesterol depletion from NIH3T3 cells bearing HIV-1 receptors (NIH3T3CD4R5/NIH3T3CD4X4) did not impair subsequent fusion with HeLa cells expressing the corresponding HIV-1 Envs. In contrast GSL depletion from these targets reduced fusion by 50% suggesting that GSL facilitate fusion in different ways. (3) GSL-deficient GM95 cells bearing high receptors fused with HIV-1 Env-expressing cells at 37°C with kinetics similar to that of GSL + NIH3T3 targets. Based on these observations, we propose that the plasma membrane cholesterol is required to maintain the integrity of receptor pools whereas GSLs are involved in stabilizing the coupling of inter-receptor pools.

Keywords Viral entry · Cholesterol · Sphingolipids · Membrane fusion · HIV-1

Abbreviations

CT	cholera toxin
GSL(s)	glycosphingolipid(s)
SM	Sphingomyelin
MeβCD	methyl β-cyclodextrin
HIV-1 Env-gp	human immunodeficiency virus Type-1 Envelope Glycoprotein
HIV-1	human immunodeficiency virus Type-1
DRMs	detergent-resistant membrane domains
Lipid Rafts	dynamic assemblies of lipids and proteins in the plasma membrane of cells enriched in sphingolipids and cholesterol, which are characterized by virtue of their insolubility in Triton X100 at 4°C also termed as detergent resistant membranes (DRM)

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Introduction

HIV-1 infects host cells by fusion of viral and target membranes. This fusion event occurs at the physiological pH and is triggered by sequential interaction(s) of HIV-1 fusion protein, gp120-gp41 (HIV-1 Env-gp), with cellular CD4 and the chemokine receptors CXCR4 or CCR5 [1,2]. Both lipids and proteins play critical and cooperative roles in the fusion process. Lipids contribute in the viral fusion process by virtue of their distinct chemical structure, composition and/or their preferred partitioning into specific microdomains in the plasma membrane called "rafts." An involvement of lipid rafts in HIV-1 entry and membrane fusion has been examined (see review [3]). However, the mechanism(s) by which lipids as dynamic raft components control HIV-1 envelope-glycoprotein-triggered fusion is not clear.

GSLs have also been proposed as attachment factors for initial capture of HIV-1 by intestinal epithelial cells. Bomsel *et al.*, reported that the infectious HIV could cross a tight human epithelial cell barrier by transcytosis [4]. An involvement of target membrane GSLs [5,6] is based on the

fact that inhibition of target cell GSL biosynthesis reduces HIV-1 fusion, and that fusion activity can be recovered following addition of purified GSLs (such as Gb3 and GM3) to the impaired cells [5,6]. Moreover, studies using monolayers of purified GSLs provide evidence for CD4-induced interactions between HIV-1 Env and the GSLs, Gb3 and GM3 [7,8]. Interactions between HIV-1 and HIV-2 envelope glycoproteins and specific GSLs, GalCer, Gb3, and GM3 were documented by measuring variations in surface pressure of GSL monolayers [8,9]. A similar study showed preferential interaction of Gb3 and GM3 with CXCR4- and CXCR4/CCR5-utilizing gp120s respectively [8]. These observations have led to the hypothesis that plasma membrane GSL-enriched microdomains are preferential sites for assembly of the HIV-1 fusion machine [8].

Recently, Conboy *et al*. [10] have done quantitative measurements of the interaction of certain GSLs with recombinant HIV-1 gp120. This study compared the binding affinities of LacCer, GlcCer and GalCer to gp120. Among these, only GalCer had high affinity for gp120 (Ka $= 1.6 \times 10^9$ M-1). Gp120 did not show significant affinity for either GlcCer or LacCer. Moreover, when the fatty acyl chain length of GalCer was varied from C18 to C24 no effect on the binding affinity of GalCer to gp120 was observed, suggesting that the binding depends more on the head group than on the chain length. Studies of GSL-gp120 interactions in model systems have largely been done using monomeric gp120. Gp120, as expressed in HIV-1, exists however as a trimer. It remains to be studied whether the trimeric nature of gp120 has any effect on the binding affinities with the GSLs.

An involvement of target membrane cholesterol in HIV-1 entry and membrane fusion has been previously demonstrated [11,12]. Treatment of target cells with a reagent, methyl-β-cyclodextrin (MeβCD), which is known to deplete cholesterol from the plasma membrane, blocked HIV-1 infection and membrane fusion with HIV-1 Env-expressing cells [12]. Both infection and fusion were recovered by addition of cholesterol to depleted cells [12]. However, the removal of cholesterol from cells expressing high CD4 and chemokine receptor levels does not inhibit subsequent fusion with the corresponding HIV-1 Env-expressing cells [13]. Therefore, in contrast to the recently reported structural requirements of cholesterol in the membrane of HIV-1 particles [14–16], specific interactions of target membrane cholesterol with HIV-1 Env and/or receptors do not appear to play a role in HIV-1 entry.

Our recent findings using the GSL-deficient mutant GM95 cells show that GSLs are required at low receptor levels, but fusion can occur provided receptors are expressed at relatively high levels in the absence of target membrane GSLs [17]. These observations suggest that GSLs promote fusion by recruitment of sufficient receptors when receptor levels in the target membrane are limiting. However, GSL depletion from GSL+ NIH3T3 target cells (which express high receptors) reduced fusion suggesting multifaceted roles of GSLs for HIV-1 fusion. To resolve these seemingly contradictory effects, we will confine ourselves to the experiments using the GSL+ and GSL− target cells, which express high level of surface receptors. We will further elaborate on the possible mechanisms by which these two raft-associated lipids promote CD4-mediated HIV-1 entry. Our observations that NIH3T3 cells expressing high CD4 and co-receptors were only sensitive to GSL depletion suggest that GSLs promote fusion by coupling inter-receptor pools at low density of receptors, whereas cholesterol is required to organize the multimeric envelope/receptor complexes in clusters.

Materials and methods

Materials

Fluorescent probes were obtained from Molecular Probes (Eugene, OR), and tissue culture media were obtained from Gibco/BRL (Gaithersburg, MD). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and d,l-threo-l-Phenyl-2-hexadecanoylamino-3-morpholino-propanol·HCl (PPMP), GSLs, and the monosialoganglioside mixture were from Matreya (Pleasant Gap, PA). Other reagents were from Sigma (St Louis, MO). vPE16 [18] and vCB43 [19] were gifts from Drs. P. Earl, C. C. Broder and B. Moss.

Cells

Source and cell culture conditions of various cell lines are described in details [6]. NIH3T3CD4X4 and NIH3T3CD4R5 (the gift of Dan Littman and Vineet KewalRamani) [20] were grown in D10 + 3 μg/ml puromycin. GM95CD4X4 and GM95CD4R5 cells were maintained in D10[17]. Sup-T1 cells (Non-Hodgkin's T-cell lymphoma cell line) [21] were obtained from the NIH AIDS Research and Reference Reagent Program. Sup-T1 cells were maintained in suspension culture using RPMI 1640 supplemented with 10% fetal bovine serum. GHOST-R3/X4/R5 (GHOST-345) were grown in D10 + 500 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml puromycin. TF228.1.16 cells (a kind gift from Zdenka L. Jonak, SmithKline Beecham Pharmaceuticals, PA) were maintained as suspension culture using RPMI-1640 supplemented with 10% heat inactivated serum and antibiotics [22]. HeLa cell lines were grown in DMEM + 10% FBS (D10). All cells were grown in the presence of penicillin and streptomycin.

HIV-1 envelope glycoprotein expression

HIV envelope proteins were transiently expressed on the surface of HeLa cells using the recombinant vaccinia constructs vPE16 (HIV-1IIIB, X4-utilizing) and vCB43 (HIV-1BaL, R5-utilizing) as described previously [23]. Alternatively, we used TF228 cells, a B lymphocyte cell line which constitutively expressing HIV-1IIIB envelope.

GSL depletion by treatment with PPMP

Target cells were incubated with the 7.5 or 10 μM GSL biosynthesis inhibitor, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) before fusion assay essentially as described [6,24]. Cells were grown in medium containing PPMP for 3 to 7 days before being used in a fusion assay. GSL down-modulation was assessed by lipid analysis essentially as described [25]. Immunostaining with FITC-conjugated cholera toxin (which specifically binds to GM1) by microscopy or flow cytometry was performed on Sup-T1 cells, which express GM1 as one of the major gangliosides.

Cholesterol depletion from target cells

Cholesterol depletion and subsequent addition to the target cells was done using methyl-β-cyclodextrin as described [26]. Cholesterol levels in the cells were assayed using the cholesterol assay kit (Molecular Probes Inc., Eugene, OR).

HIV-1 envelope glycoprotein-mediated cell-cell fusion

Cell-cell fusion between CD4+ targets and HIV-1 Env-expressing effector cells was assayed using the fluorescent dye transfer method [27]. Typically target cells were plated on 12 well clusters at a density of 50,000 cells/well one day prior to fusion experiments and were labeled with the cytoplasmic dye CMTMR (green emission, ex/em 540/566 nm), at a concentration of 5–10 μM for 1 h at 37°C according to manufacturer's directions. Alternatively, target cells were metabolically labeled with the fluorescent fatty acid (4,4-difluoro-5-(2-thienyl)-4-bora-3a, 4a-diaza-*s*-indacene-3-dodecanoic acid (BODIPY® 558/568 C12, Molecular Probes, Product #: D-3835)) as follows: Cells were seeded at 10^5 cells/ml in medium containing 10 μg/ml of the BODIPY-C12 and grown for three days prior to experiment (Molecular Probes, Eugene, OR). The fatty acid was eventually metabolized into phospholipid, primarily phosphatidylcholine (data not shown). The HIV-1 Env- expressing cells were labeled with either calcein or CMFDA following manufacturer's directions. For calcein labeling, cells were incubated with 1 μg/ml calcein for 60 min at 37°C, and then washed and resuspended at 10^5 cells/ml in the culture medium. To label with CMFDA, cells were incubated with 10 μM CMFDA (blue emission, ex/em 492/516 nm) for 1 to 2 hrs at 37°C. Fluorescently labeled effector and target cells were co-cultured under various experimental conditions. Details of specific culture conditions of the specified experiments are described in the figure legends. Dye redistribution was monitored microscopically as described previously [5]. The extent of fusion was calculated as Percent Fusion = 100 × number of bound cells positive for both dyes/number of bound cells positive for CMTMR (or Red-BODIPY). When fusion assays were performed on PPMP-treated cells, all media contained d-l-, threo-PPMP.

Results

PPMP pre-treatment of Sup-T1 cells blocks HIV-1IIIB-mediated fusion

Our previous studies show that PPMP-treatment of adherent cell lines bearing cognate HIV-1 receptors inhibits HIV-1 Env-mediated membrane fusion and entry. Since HIV-1 is known to infect circulating CD4+ lymphocytes, we evaluated the involvement of GSL in HIV-1 Env-mediated membrane fusion in Sup-T1 cells (a Non-Hodgkin's T-cell lymphoma cell line which expresses high levels of surface CD4). Sup-T1 cells were incubated in the presence of a GSL-biosynthesis inhibitor, PPMP, which is known to down-regulate GSLs and block HIV-1 entry and fusion. GSL depletion in PPMP-treated Sup-T1 cells was confirmed by staining with FITC-conjugated CT, which specifically binds to GM1 (one of the major gangliosides of Sup-T1cells and T lymphocytes). Figure 1C shows that GM1 levels were significantly reduced from the surface of Sup-T1 cells after treatment with PPMP. The quantitative differences in GM1 levels were further determined by FACS (Fig. 1B). It is evident from the results, that surface-expressed GM1 levels were significantly reduced in PPMP-treated Sup-T1 cells (Fig. 1C). Next, we investigated the effect of GSL depletion from Sup-T1 cells on HIV-1IIIB-mediated fusion using the fluorescent dye-transfer assay [27]. When PPMP-treated Sup-T1 cells (labeled with CMTMR) were incubated with calcein-labeled TF228 cells (a B lymphocyte cell line which constitutively expresses HIV-1IIIB Env), we observed a significant decrease in fusion (70% reduction compared to untreated cells, Fig. 1A). The values expressed are an average of at least three samples within a single experiment. Results presented here show that PPMP treatment of lymphocytic cells decreases GSLs and blocks HIV-1 Env-mediated fusion, similar to the effect observed when adherent cell lines were used as targets.

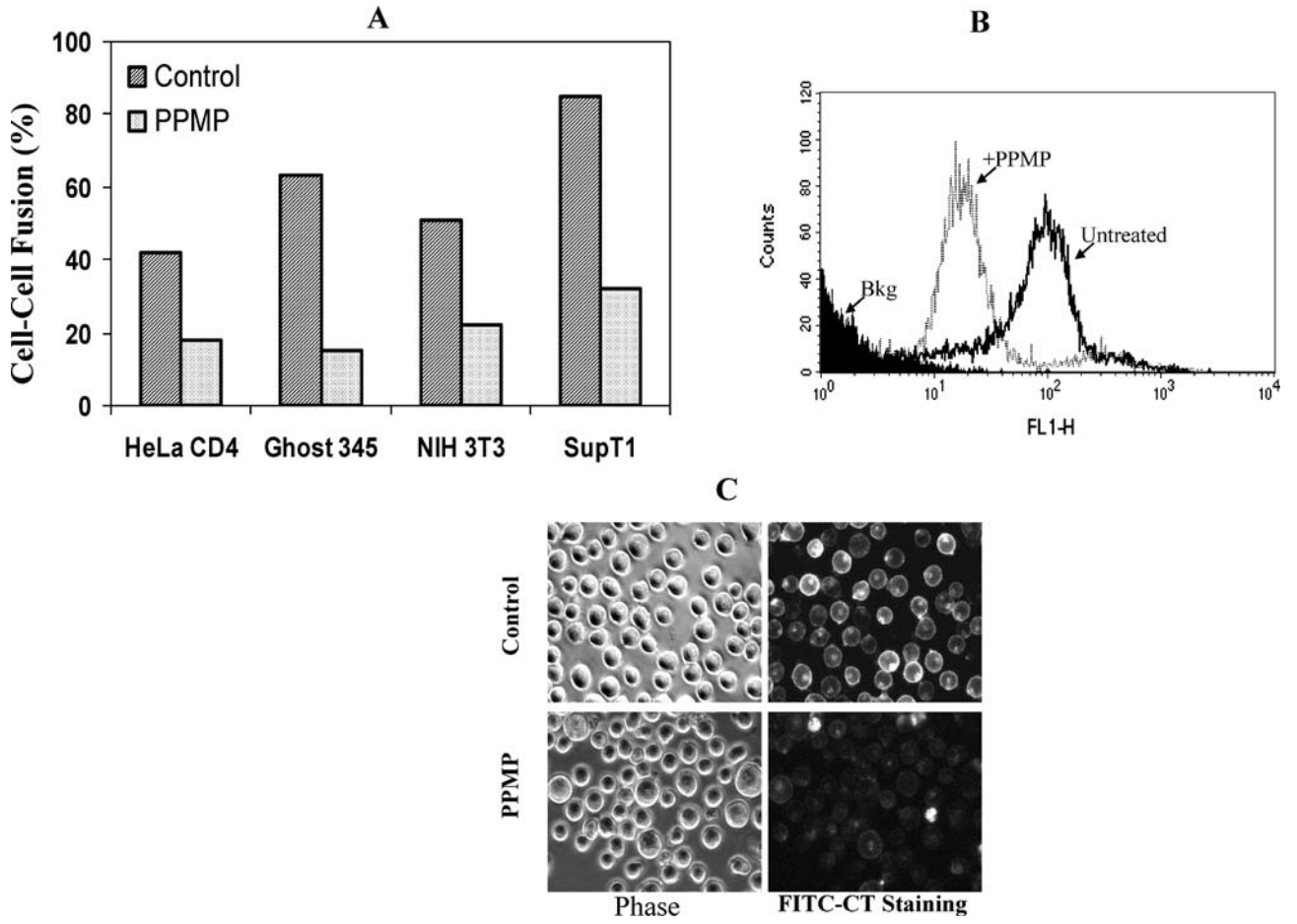

Fig. 1 Effect of PPMP Treatment of CD4, CXCR4 and/or CCR5-expressing cells on HIV-1 Env-mediated Fusion. Control and d-l-threo-PPMP treated cells were labeled with CMTMR. HeLaCD4, NIH3T3, and Ghost 345 cells were then cultured for two hrs at 37°C with Calcein-AM-labeled TF228 cells, while Sup-T1 cells were incubated with HIV-1 Env-expressing cells (Fig. 1A). The Fig. 1B shows the effect of PPMP treatment on GM1 levels of Sup-T1 cells. For GM1 immunostaining, cells (10^6/0.1 ml) were treated with 3–5 μg FITC-CT (B subunit, Sigma Chemical Co, St. Louis, MO) in PBS-BSA for 60 min at 40°C. Unbound FITC-CT was removed and cells were analyzed by FACS (top, right panel) or by microcopy using 40X oil lens (1.4 NA) (Fig. 1C)

Target membrane GSLs do not modulate kinetics of HIV-1 fusion at high receptor expression

Our recent studies using a GSL-deficient mutant mouse cell line (GM95) show that target membrane GSLs are required at relatively lower expression of receptors. Although GM95 cells became susceptible to fusion only when receptors were expressed at relatively high levels, fusion occurred at 25°C and was more sensitive to membrane cholesterol when compared with GSL+ NIH3T3 targets [17]. These findings suggested that GSLs also promote HIV-1 fusion by stabilizing the envelope-receptor complexes.

To further examine this issue, we compared kinetics of fusion of GSL-deficient GM95 cells with the GSL+ NIH3T3 targets, which express high levels of HIV-1 receptors. Fusion activity of GM95 and NIH3T3 cell lines during 2 h of incubation at 37°C is shown in Fig. 2. It is clear from the micrographs that robust fusion (appearance of multi-nucleated cells) occurred independent of the target cells used. Fusion was further confirmed by colocalization of red/green dyes in fused cells. These results are consistent with our recent findings [17]. Next, we monitored time-dependent kinetics of cell-to-cell fusion at 37°C [27]. Figure 3A shows that the GM95CD4R5 cells began to fuse at 20 min post-coculture, and fusion reached a plateau at 60 min (Fig. 3A, solid squares). Kinetics of fusion of GM95CD4R5 cells (Fig. 3A, solid squares) was very similar to that for the NIH3T3CD4R5 cells (Fig. 3A, open diamonds) indicating that at 37°C, GSL do not affect kinetics of HIV-1 fusion. We observed a delay in kinetics of fusion of GM95CD4X4 cells with HIV-1IIIB-expressing HeLa cells as compared to the NIH3T3CD4X4 targets (Fig. 3B). We attribute this delay in kinetics of GM95

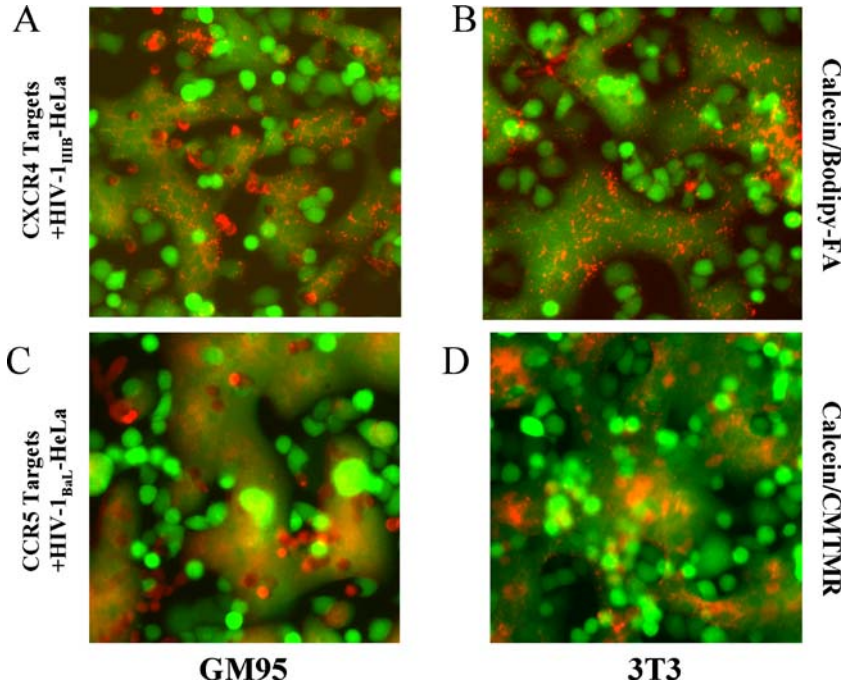

Fig. 2 Fusion activity of GSL-deficient GM95 cells bearing HIV-1 receptors. CD4 and CXCR4, bearing target cells were metabolically labeled with red BODIPY-FA (see text) and then plated on 12 well clusters at a density of 5×10^4 cells/well one day prior to fusion experiments. The target cells were incubated with calcein labeled HIV-1IIIB Env-expressing HeLa cells for 2 h at 37°C and images were captured. The results are shown in the top panel as indicated. CD4- and CCR5-bearing targets were plated on 12-well plates (5×10^4 cells/well) and were labeled with the cytoplasmic dye CMTMR. Calcein-labeled HIV-1BaL Env-expressing cells were incubated with the CMTMR-labeled CD4, CCR5-bearing targets for two hrs at 37°C. Images were collected (see text). The results are shown in the bottom panel (Fig. 2) as indicated

cells to relatively reduced CD4 and CXCR4 expression in GM95 cells in comparison to the NIH3T3 cells (not shown). Taken together, results presented in Fig. 3 show that at a high density of receptors, target membrane GSLs do not modulate the kinetics and/or extent of HIV-1 Env-mediated fusion.

GSL but not cholesterol depletion from NIH3T3 cell-bearing high receptors blocks fusion

Our recent studies show that HIV-1 Env-mediated fusion of GM95 cells was dependent on the membrane cholesterol in

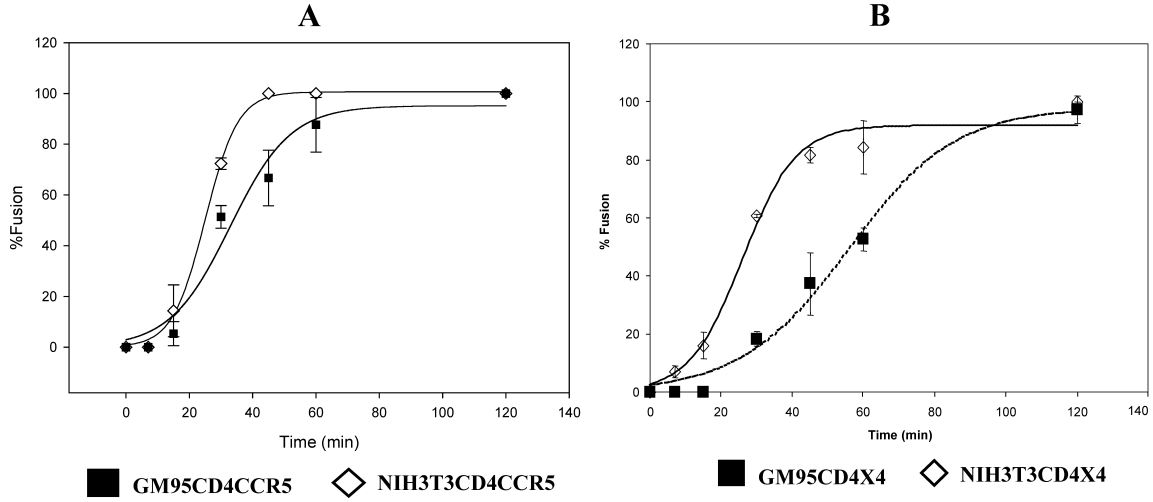

Fig. 3 Kinetics of fusion. Target cells were plated on 12-well tissue culture plates at 10^5 cells per well and were labeled with the cytoplasmic dye CMTMR. Calcein-labeled HIV-1 Env-expressing cells (1×10^6 per well) were added to the wells containing target cells and incubated for various amounts of time in a 37°C incubator. At the end of incubations, images were collected using a 10X objective, and fusion was scored using the assay described in the text. Panel A shows the fusion kinetics of GM95 cells (solid squares) and NIH3T3 (open diamonds) cells expressing CD4 and CCR5. Panel B shows the GM95 cells (solid squares) and NIH3T3 (open diamonds) CD4 and CXCR4. We attribute the slower fusion kinetics of GM95 cells expressing CD4 and CXCR4 to lower expression of co-receptors in comparison to NIH3T3 cells

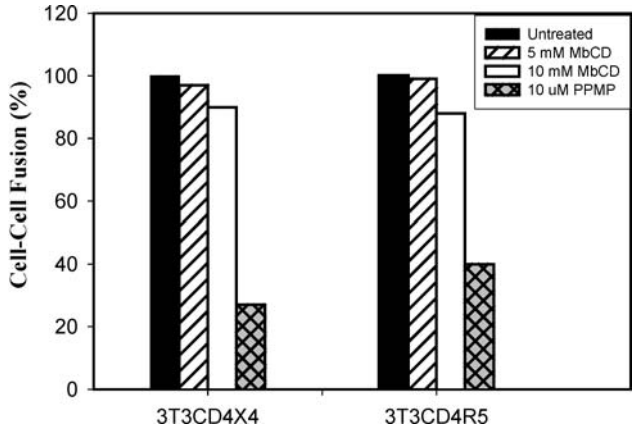

Fig. 4 Effect of cholesterol and GSLs depletion from receptor-bearing NIH3T3 cells on HIV-1 Env-mediated fusion. Target cells on 12-well plates were labeled with CMTMR and then treated with 5 or 10 mM MeβCD for 30 min at 37°C essentially as described CMFDA-labeled HIV-1 Env-expressing cells were added to the matching targets, and the samples were incubated at 37°C for 2 to 4 hrs. Fusion activity was monitored using HeLa cells infected with vaccinia vectors, which express HIV-1 Env from a CXCR4-utilizing isolate (IIIB) and a CCR5-utilizing isolate (Ba-L). The percentage of fusion was determined as described in the text

contrast to that of the NIH3T3 cells bearing high receptors. We attributed these differences to the absence of GSLs in GM95 cells. However, possible contribution of target membrane components other than GSLs for this cholesterol sensitivity cannot be ruled out. Therefore, we compared fusion activity of NIH3T3CD4X4 or NIH3T3CD4R5 cells after removal of GSL or cholesterol. Results from these experiments are presented in Fig. 4. Preincubation of NIH3T3 cells with 10 μM PPMP for 48 to 72 h at 37°C resulted in a substantial decrease in fusion (70 to 80%) consistent with our previous findings [6]. However, removal of cholesterol by treatment of MeβCD did not have a significant effect on fusion (only 10% decrease as compared to untreated cells). Therefore, clearly GSLs promote HIV-1 fusion in multiple ways.

Discussion

HIV-1, a member of lentivirus family, gains entry into susceptible cells by fusion of the viral membrane with the cell plasma membrane [28,29]. This process is mediated by the interaction of the HIV-1 envelope (Env) glycoprotein with CD4 on the host cell surface and requires coreceptors, such as CXCR4 or CCR5, that determine the tropism of different HIV-1 isolates [30]. Several viral envelope glycoprotein oligomers then assemble into a viral fusion machine [31,32], forming a molecular scaffold that brings the viral and target cell membranes into close apposition and precipitates the actual fusion event [33]. In recent years, several groups have focused their efforts to investigate an involvement of the target membrane cholesterol [34] and sphingolipids [6] in HIV-1 entry and fusion.

The inhibition of CD4 and coreceptor-dependent HIV-1 fusion in adherent cell lines following reduction of GSLs has been reported previously [5,6]. Our observations that GSL depletion from Sup-T1 cells (a suspension T cell line naturally expressing CD4 and CXCR4) also blocked HIV-1IIIB Env-mediated fusion further support the role of GSLs in HIV-1 entry in natural targets [35]. Our cell-cell fusion kinetics studies with GM95 target cells did not reveal any significant effect on fusion in the absence of GSLs; therefore, we propose that at high receptor expression, receptor/envelope recruitment to the fusion site is not the limiting step in fusion [17]. Since GM95 cells expressing low receptors did not promote fusion, GSL may also assist in receptor recruitment of receptors in natural targets [17].

In the light of current and previous findings, one can depict that GSLs play an auxiliary function via random as well as specific associations. GSLs may (1) enhance virus attachment by direct interactions with the HIV-1 envelope, CD4, and/or chemokine receptors, (2) be involved in clustering of sufficient receptors to the fusion site, or (3) promote a long-lived state of the activated HIV-1 envelope. Target membrane cholesterol appears to provide the necessary membrane organization and appropriate milieu for fusion to occur. Specific interactions of target membrane cholesterol with the viral envelope and/or receptors are not required for HIV-1 entry [13]. This is in contrast to the structural requirement of cholesterol in the virus membrane for infection [15,16].

To explain the role of the two raft-associated lipids, GSLs and cholesterol in HIV-1 entry, we propose the "Traffic Light Model (TLM)" (Fig. 5). According to the model, expression of high levels of CD4 and/or CXCR4 in the membrane allows cooperative interactions leading to fusion, in the absence of GSLs. However, at physiological levels of CD4 and CXCR4, GSLs allow enrichment of CD4 and CXCR4 in GEMs. PPMP treatment of the cells reduces cellular GSLs and, therefore, CD4/CXCR4 clustering is impaired which results in inhibition of fusion.

Initial interaction between HIV Env trimers (red) and CD4 molecules (cyan) results in the triggering of HIV Envs (orange) so that binding sites on gp120 become available for coreceptor engagement [23]. The activated Envs (yellow) then need to take hold of a cluster of co-receptors (magenta) to trigger the fusion reaction (green). The probability of coreceptor engagement is enhanced by localization of CD4 clusters at a reasonable distance from co-receptor clusters [26,36]. At high co-receptor surface densities, GSLs are not required since activated Envs will readily find co-receptors in their vicinity. However, at low surface density of co-receptor, GSLs (brown) may be required to hold activated Env for a sufficient amount of time to scan the sur-

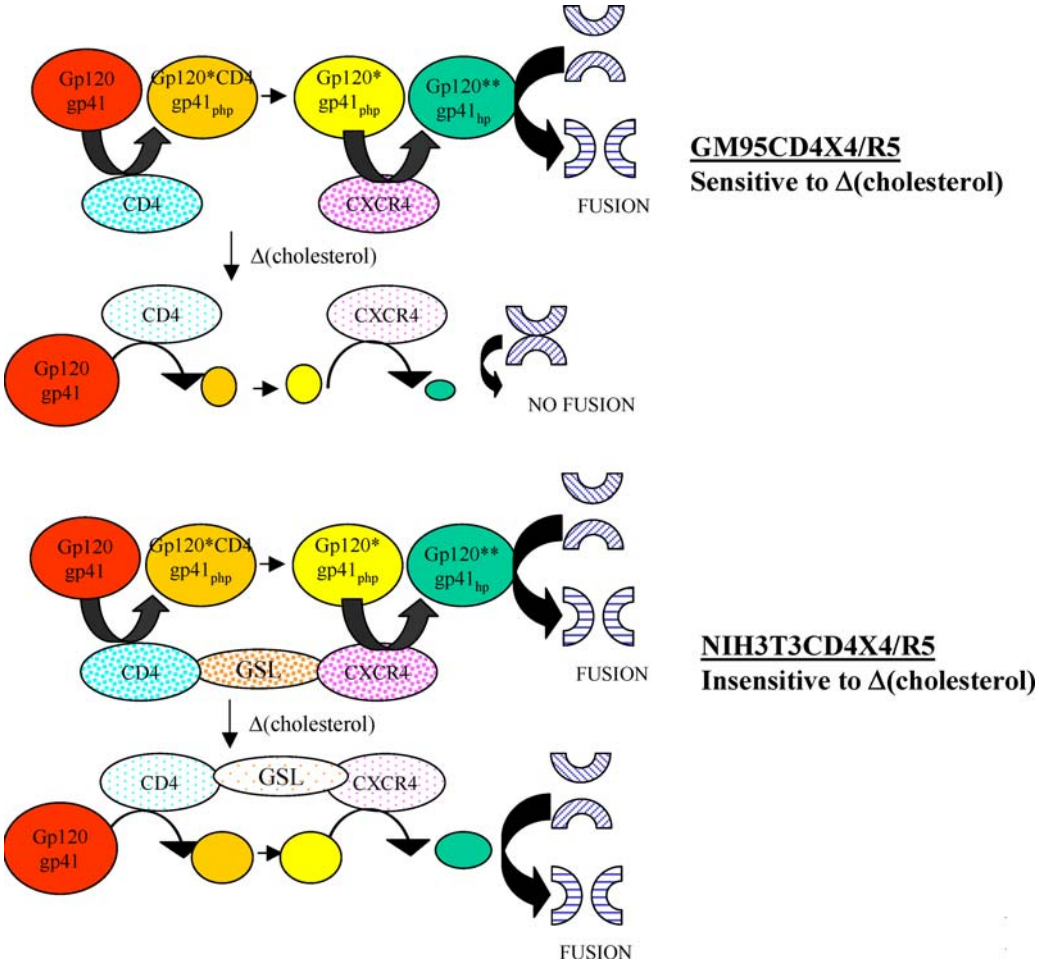

Fig. 5 A Model presenting the role of target membrane GSLs and cholesterol in HIV-1 Env-mediated fusion. See text (Discussion section) for details (colored figure available online)

face and find co-receptors needing to be engaged. Studies on binding of gp120 to GSL are consistent with this notion [37].

The effects of lowering cholesterol levels may lead to a "phase separation" [38,39] of CD4 domains and co-receptor domains. Similar effects may be caused by blocking F-actin polymerization [40]. As a consequence CD4-triggered Env becomes less capable of engaging coreceptors. Presence of GSL on cells may mitigate the effects of cholesterol removal by reducing the phase separation and by holding triggered Env for a longer time in a conformation that is still capable of engaging co-receptor. A prediction of this model is that Env will be more rapidly inactivated by its interaction with CD4 and coreceptor in the absence of GSLs.

How could GSLs play a role in HIV-1 entry? Studies suggest sphingolipid and cholesterol-rich domains may exist in the membrane as phase-separated "rafts", which serve as sites enriched in signal transduction assemblies [41,42]. According to a previous model proposed by Fantini *et al.* [8] the glycosphingolipids recognized by both CD4 and gp120 induce the formation of a trimolecular complex of CD4, glycosphingolipids and gp120 in such rafts. The observation that CD4 is found in GM3−enriched domains on the lymphocyte plasma membrane [43,44] supports this hypothesis. Further experiments are needed to test the validity of this hypothesis. Therefore, GSL can promote HIV-1 fusion by stabilizing the association between HIV-1 Env, CD4 and/or the coreceptor via specific interactions.

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