Raft localization of CXCR4 is primarily required for X4-tropic human immunodeficiency virus type 1 infection

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Abstract

Human immunodeficiency virus type 1 (HIV-1) infection is initiated by successive interactions of viral envelope glycoprotein gp120 with two cellular surface proteins, CD4 and chemokine receptor. The two most common chemokine receptors that allow HIV-1 entry are the CCR5 and CXCR4. The CD4 and CCR5 are mainly localized to the particular plasma membrane microdomains, termed raft, which is rich in glycolipids and cholesterol. However, the CXCR4 is localized only partially to the raft region. Although the raft domain is suggested to participate in HIV-1 infection, its role in entry of CXCR4-tropic (X4-tropic) virus is still unclear. Here, we used a combination of CD4-independent infection system and cholesterol-depletion-inducing reagent, methyl-β-cyclodextrin (MβCD), to address the requirement of raft domain in the X4-tropic virus infection. Treatment of CD4-negative, CXCR4-positive human cells with MβCD inhibited CD4-independent infection of the X4-tropic strains. This inhibitory effect of the cholesterol depletion was observed even when the CXCR4 was over-expressed on the target cells. Soluble CD4-induced infection was also inhibited by MβCD. The MβCD had no effect on the levels of cell surface expression of CXCR4. In contrast to these infections, MβCD treatment did not inhibit CD4-dependent HIV-1 infection in the wild type CD4-expressing cells. This study and previous reports showing that CD4 mutants localized to non-raft domains function as HIV-1 receptor indicate that CXCR4 clustering in the raft microdomains, rather than CD4, is the key step for the HIV-1 entry.

Introduction

Human immunodeficiency virus type 1 (HIV-1) gains entry into susceptible cells by fusion of the viral membrane with the cell plasma membrane (Dimitrov, 2000). This process is generally initiated by the binding of the HIV-1 envelope (Env) glycoprotein gp120 to CD4 on the host cell surface. The binding then induces conformational change of the gp120, which allows gp120 to interact with a cellular surface chemokine receptor, termed coreceptor. HIV-1 can use many types of chemokine receptors for the entry (Shimizu et al., 2000). The two most common types of the coreceptors of the HIV-1 are the CC chemokine receptor 5 (CCR5) and the CXC chemokine receptor 4 (CXCR4) (Berger et al., 1999). Successive conformational changes in the gp120 during these interactions with cellular surface molecules render initially occluded hydrophobic domain of the envelope gp41 subunit available to fusion with cellular plasma membrane (Doms, 2000).

Clustering of multiple CD4 and coreceptor molecules at the site of the fusion is presumed to be necessary for the efficient fusion of the viral and host cell membranes (Kuhmann et al., 2000). Because both the gp120-CD4 and gp120-chemokine receptor associations are reversible, and because CD4 binding site of the gp120 is conformationally masked (Kwong et al., 2002), multiple CD4 and chemokine receptor molecules should almost simultaneously be gathered and interact with the multiple gp120 at the place of virus-host cell membrane fusion (Dimitrov 2000; Doms, 2000; Kwong et al., 2002).

Membrane microdomains or lipid rafts are regions of host cell membrane enriched in glycosphingolipids, sphingomyelin, cholesterol, glycophosphatidylinositol-anchored proteins, and signaling proteins (Simons and Ikonen, 1997). The rafts are thought to serve as sites for recruitment of gp120-gp41-CD4-coreceptor complexes in a limited area on the cell surface. Increasing evidences indicate

such a scaffolding role of the rafts in HIV-1 entry; (i) HIV-1 infection is blocked by targeting CD4 to non-raft membrane domains (Del Real et al., 2002); (ii) membrane raft microdomains mediate lateral assemblies required for HIV-1 infection (Manes et al., 2000); (iii) HIV-1 gp120-induced co-clustering of CD4 and coreceptor into the raft domains is prevented by removal of cholesterol from cell plasma membrane and the depletion of cholesterol from target cells inhibits their susceptibility to HIV-1 infection (Manes et al., 2000; Popik et al., 2002; Liao et al., 2001; Viard et al., 2002).   Together with other results, reported data are compatible with the possibility that the recruitment of gp120-gp41-CD4-coreceptor complexes into the raft domains is required for the HIV-1 infection (Liao et al., 2001; Manes et al., 2000; Popik et al., 2002).   However, it is not clear what is the determinant for the recruitment of the complexes into raft domains.

CD4 (Millan et al., 1999; Manes et al., 2000; Del Real et al., 2002) and CCR5 (Nguyen and Taub, 2002b; Gaibelet et al., 2006) have been demonstrated to be present in lipid rafts, and to constitutively interact each other before the gp120 binding.   In contrast, CXCR4 is localized only partially to the rafts, as evidenced with partial colocalization with GM1, a raft marker (Manes et al., 2000; Del Real et al., 2002; Nguyen and Taub, 2002a).   It has been reported that a CD4 mutant, which is localized to non-raft domains of the plasma membrane, blocks HIV-1 entry, indicating that raft localization of CD4 is critical in HIV-1 infection (Del Real et al., 2002).   However, more recent studies are consistent with a possibility that the raft localization of CD4 is not required for the virus entry (Popik and Alce, 2004; Percherancier et al., 2003), indicating that CD4 is not the determinant for clustering of gp120-gp41-CD4-chemokine receptor complexes into raft domains.   Due to the initial localization of CXCR4 in the non-raft region and the inconsistencies in prior studies, a role of raft domains in CXCR4-tropic (X4-tropic) HIV-1 entry is not clear yet.

These apparently controversial observations prompted us to examine the possibility that recruitment of CXCR4 to raft microdomains, rather than CD4 raft localization, is the determinant for the clustering of gp120-gp41-CD4-CXCR4 complexes into raft domains. To test the possibility, we used HIV-1 pseudotype viruses that have the X4-tropic Env proteins and can establish infection of CXCR4-expressing cells without interaction with CD4 (CD4-independent infection). The viruses were used to infect cells whose cholesterol were depleted in advance with treatment by a cholesterol–solubilizing agent, methyl-β-cyclodextrin (MβCD) (Simons and Ikonen, 1997), and viral infectivity was measured. We further examined a role of raft localization of CXCR4 in the HIV-1 entry, as follows. The CD4-dependent infection induced by soluble CD4 was used to infect cholesterol-depleted cells. By this approach, we can determine if the raft localization of CXCR4 is essential in the HIV-1 infections, because these infections occur independently of CD4 raft localization. Our results are compatible with the working hypothesis described above and suggest a supportive role of CD4 in augmenting the raft recruitment/clustering of CXCR4.

Materials and Methods

Env protein expression plasmids. The CD4-independent HIV-1 Env (mNDK strain) and its parental CD4-dependent HIV-1 Env (NDK strain) expression plasmids were kindly obtained from Dr. U.Hazan (Dumonceaux et al., 1998). The CD4-independent HIV-1 Env (8X strain) expression plasmid was kindly provided from Dr. R. Doms (Hoffman et al., 1999). The CD4-dependent HIV-1 (HXB2 strain) Env expression plasmid was kindly obtained from Dr. Y. Yokomaku. The VSV-G expression plasmid (pHEF-VSVG) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAIS, NIH, USA (Chang et al., 1999).

Cells. Human glioma NP2 (Soda et al., 1999), human rhabdosarcoma TE671, human cervical cancer HeLa, and human embryonal kidney (HEK) 293T cell were cultured in Dulbecco's modified Eagle's medium (Sigma) at $37^{\circ}C$ in 5% CO_2. The culture media were supplemented with 8% fetal bovine serum (Biofluids). The original NP2 cells do not express endogenous CXCR4 and CD4 proteins, whereas NP2/X4 and NP2/CD4/X4 cells were processed to express exogenous CXCR4 alone and both of CD4 and CXCR4, respectively (Soda et al., 1999). TE671/CD4 and 293T/CD4 cells were constructed as follows. HEK 293T cells were transfected with murine leukemia virus (MLV) *gag-pol* (3µg) (TAKARA), CD4-encoding retroviral vector (3µg), and VSV-G expression plasmid (3µg) (Chang et al., 1999) by the TransIT LT1 regent (30 µl) (Mirus). The cells were washed 24 hr after transfection, and cultured for 24 hr in fresh medium. Culture supernatant of the transfected cells was inoculated into 293T or TE671 cells. The inoculated cells were selected by puromycin (10 µg/ml). The puromycin-resistant cell pool was utilized in this study. TE671 and 293T cells over-expressing CXCR4 were constructed by transduction of these cells with CXCR4-encoding MLV vector as

described above.

Transduction assay.   To obtain HIV-1 vector particles containing Env protein, human 293T cells were transfected with a packaging construct of HIV-1 (R8.91) (Naldii et al., 1996), LacZ-containing HIV-1 vector (Iwakuma et al., 1999), and the appropriate Env expression plasmids (3 μg each) by the Trans IT-LT1 reagent.   The transfected cells were washed with medium 24 hr after transfection, and continued to be cultured in fresh medium for 24 hr.   Target cells were inoculated with the culture supernatants of the transfected cells in presence or absence of soluble CD4 (20 μg/ml) (obtained from AIDS Research and Reference Reagent program, NIH).   The inoculated cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Wako) 2 days after inoculation.   Blue cells were counted to estimate transduction titer.   Undiluted CD4-independent and -dependent vectors induced usually around 5×10^2 and 5×10^4 blue cells.   Therefore, in this study, CD4-dependent vectors were diluted 100 times with medium.

Cholesterol depletion.   The cells were treated with methyl-β̄cyclodextrin (MβCD) (Sigma) in FBS-free medium, or with 5mM MβCD and 50 μg/ml cholesterol (Wako) for 30-120 min at 37℃.   As control, the cells were exposed to FBS-free medium alone.   After incubation, the cells were washed with phosphate buffer saline (PBS) before being used to remove MβCD and cholesterol.

Filipin Staining.   The treated cells were fixed with 1% p-formaldehyde in PBS for 10 min at room temperature.   The fixed cells were washed and stained with filipin (0.1 g/ml) (Sigma) in PBS for 2 hr at room temperature.   After washing with PBS, the cells were collected by scraper and fluorescence strength at 525 nm of the cells was analyzed by a flow cytometry (Becton Dickinson).   Fluorescence strength of filipin at 525 nm is reduced by its binding to cholesterol (Severs et al., 1983; Castanho et al., 1992).

FACS. To analyze cell surface expression of CXCR4, suspended cells were treated with a rat anti-CXCR4 antibody (A80) for 1 hr at 4℃. The CXCR4 antibody (A80) recognizes the third extracellular loop of CXCR4 (Tanaka et al., 2001). The cells were washed with PBS 3 times, and then treated with a FITC-conjugated anti-rat IgG antibody (Sigma). The stained cells were applied to a flow cytometry (Coulter).

Assay of HIV-1 Env-mediated cell fusion. HEK 293T cells were transfected with the HIV Env expression plasmid. The plasmid additionally encodes the tat protein. HEK 293T cells were transfected with a tat expression plasmid as a control. Target cells were transfected with the LTR-LacZ plasmid, and then were treated with MβCD. These cells were mixed 48 hr after transfection, and cultured for 24 hr. LacZ activities of the cell lysates prepared from the mixed cultures were measured by the high sensitive β-gal activity measurement kit (Stratagene).

Fractionation of raft membrane microdomains. HA-tagged CXCR4 and CD4 expressing cells were washed and lysed on ice by 0.1% Triton X-100. The cell lysates were centrifuged and its supernatant was defined as soluble fraction. Equal volume of sample buffer was added to its precipitates, and it was defined as insoluble fraction. These fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%), and were transferred onto a PVDF membrane (Millipore). Western immunoblotting was performed using a mouse anti-HA monoclonal (Convance) or anti-CD4 antibody (Santa Cruz Biotechnology). A horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Red) was used as secondary antibody. Antibody-binding proteins were visualized using ECL Western blotting detection regents (Amersham Phaemacia Biotech).

Immunofluorescence microscopy. CXCR4 expressing cells were cultured on four-well

culture slides (Miles) for 24 hr.   Cells were incubated with the rat anti-CXCR4 antibody (A80) for 1 hr at 4℃,

followed by AlexaFlour 555-conjugated CT-B and FITC-conjugated anti-rat IgG for 1 hr at 4℃.   Cells were

observed using a confocal fluorescence microscope (Leica).

Statistical analysis.   Differences between groups of data were deter,imed by Student's t-test.

Statistical significance was set as $P<0.01$ for all tests.

Results

MβCD inhibits CD4-independent, CXCR4-dependent HIV-1 infection.

To examine whether raft domain architectures are required for CD4-dependent and -independent entry of X4-tropic viruses, we used an infection system of pseudotyped viruses carrying Env proteins of X4-tropic HIV-1 strains, mNDK, or 8X, that allow both CD4-dependent and –independent infections in CD4-positive and –negative cells, respectively (Dumonceaux et al., 1998, Hoffman et al., 1999, Kubo et al., 2007).   As targets of virus infections, we used human NP2 cells expressing both CD4 and CXCR4, or CXCR4 alone (Soda et al., 1999).   The raft domain of target cells were depleted by the treatment with MβCD (Simons and Ikonen, 1997) and used for infections of the pseudotyped HIV-1 viruses.   As a control of HIV-1 receptor independent infection, we used the VSV-G-pseudotyped HIV-1.   Incubation with MβCD did not suppress but rather increased the VSV-G-pseudotyped virus infection (Fig.1A).   This MβCD treatment (1 and 5 mM) did not affect cell growth (data not shown).

Notably, transduction titers of the HIV-1 vectors having the CD4-independent Env proteins (mNDK and 8X) were reduced to about 25% by the increasing concentrations of MβCD treatments of the CD4-negative, CXCR4-positive cells (Fig. 1B, gray bars, NP2/X4 cells).   In contrast, inhibitory effects of MβCD were less prominent in the CD4-dependent infections of the same viruses: transduction titers of the HIV-1 vectors were reduced to about 75% at 5mM of MβCD (Fig. 1B, open bars, NP2/CD4/X4 cells).   Transduction titer of the HIV-1 vector having Env protein of the NDK HIV-1 strain, the CD4-dependent parental strain of the mNDK variant, was not significantly inhibited by MβCD (Fig. 1C).   These results show that MβCD inhibits CXCR4-mediated infection but co-expression of CD4 counteracts the inhibitory effects.   When excess of

cholesterol was added, the inhibitory effect of MβCD on the CD4-independent infection was abrogated (Fig.1D),

confirming that cholesterol extraction is a primary cause of suppression of the CD4-independent infection by

MβCD.

To assess if the MβCD treatment indeed had depleted cholesterol from target cell membranes, cells

were stained with the cholesterol-binding agent, filipin. Binding of filipin to cholesterol decreases filipin

fluorescence at 525 nm (Severs and Robenek, 1983; Castanho et al., 1992). Fluorescence strength of filipin in

the MβCD-treated cells was reproductively increased compared to that of the untreated control cells (Fig.1E).

The results indicate that cholesterol of cell membrane was indeed extracted by MβCD treatment in our

experimental system.

Over-expression of CXCR4 does not affect the inhibitory effect of MβCD on CD4-independent HIV-1

infection.

In our CD4-independent infection system, virus enters into cells using endogeneously expressed

CXCR4. To know whether MβCD still inhibits infection when cells express exogenously abundant amounts of

CXCR4, human 293T and TE671 cells were transduced by an HA-tagged CXCR4 encoding murine leukemia

virus vector (Kubo et al., 2003). Over-expression of CXCR4 in the transduced cells was observed by flow

cytometry analysis (Fig.2A). Transduction titers of the CD4-independent mNDK vector in the

CXCR4-over-expressing cells increased about 3 to 4 times compared to those in the original cells (Fig.2B).

This result indicates that CXCR4-over-expression increases the susceptibility to CD4-independent infection.

Effect of CXCR4-over-expression on the inhibition of CD4-independent infection by MβCD was

analyzed.   The MβCD treatment suppressed the CD4-independent infection in the CXCR4-over-expressing

cells, as it did in the original cells (Fig. 3, upper 4 panels).   However, exogenous introduction of CD4 into the

target cells abrogated the inhibitory effect of MβCD on the HIV-1 infection (Fig. 3 lower panel), as reported

(Viard et al., 2002).   The results indicate that maintenance of the raft domain architectures on the plasma

membrane of the target cells are absolutely required for the CD4-independent infection and suggest that the raft

localization of CXCR4 is important for the HIV-1 entry.   The treatment with MβCD alone or MβCD plus

cholesterol occasionally conferred transduction titers of the VSV-G and mNDK vectors higher (Figs. 1A, 1D,

and 3).   The mechanism was not understood.

MβCD treatment inhibits CD4-independent HIV-1 Env -mediated cell-cell fusion.

The above result showed that the depletion of cholesterol by MβCD inhibited the HIV-1 Env-mediated

infection.   To examine if the depletion of cholesterol affects the HIV-1 Env-mediated cell-cell fusion, Env

expression plasmid-transfected effector cells and LTR-LacZ-transfected target cells were co-cultured (see

Materials and Methods).   The target cells were co-transfected with the Tat expression and LTR-LacZ plasmids,

and then were treated with MβCD.   LacZ activities of the cells were comparable between the cells with and

without the MβCD treatment (data not shown), indicating that the MβCD treatment does not affect LacZ

functional expression.   In NP2/CD4/X4 cells, the mNDK Env-mediated cell-cell fusion was not significantly

inhibited by MβCD (Fig.4, NP2/CD4/X4).   On the other hand, in CD4-negative NP2/X4, TE671, and 293T

cells, the CD4-independent mNDK Env-mediated cell-cell fusion was inhibited to 40 to 60 % of that in the

untreated cells (Fig.4, NP2/X4, TE671, and 293T).   The results are consistent with previous study (Alban et al.,

2006). The cell-cell fusion activity inhibited by MβCD was recovered, when excess of cholesterol was added into the culture (Fig.4, MβCD + chol). The results were compatible with those of cell-free virus infection (Figs.1 and 3). Taken together, our results suggests that cholesterol in the cell membrane played an important role in the CD4-independent HIV-1 Env-induced cell-cell fusion and virus entry.

To assess the possibility that MβCD altered the CXCR4 expression, expression levels of CXCR4 in the treated cells were analyzed by a flow cytometer. Treatment of MβCD did not affect the expression of CXCR4 in NP2/X4, 293T, and TE671 cells (Fig.5). This result indicates that the inhibition of CD4-independent infection by MβCD is not induced by reducing CXCR4 expression.

Localization of CXCR4 in rafts.

The classical way to examine the raft association of membrane proteins includes treatment of cells with Triton X–100 followed by Western blot analysis of soluble and insoluble fractions. The fractions that are not solubilized by Triton X–100 are defined as the raft membrane domains (Simons and Toomre, 2000). Using this approach, we examined localization of CXCR4 in the raft domains in the presence or absence of MβCD. We also examined raft localization of CD4 as a maker of raft protein (Manes et al., 2000; Del Real et al., 2002; Percherancier et al., 2003; Popik & Alce, 2004). CD4 levels in the insoluble fraction were higher than that in the soluble fraction in all cells examined (Fig.6A), suggesting that raft domains were correctly separated by this protocol. In contrast to CD4, CXCR4 levels in the insoluble fractions of NP2 and TE671 cells were lower than those in the soluble fractions. In contrast to these cells, CXCR4 was detected in the insoluble fraction of 293T cells. CXCR4 was detected in both of soluble and insoluble fractions after treatment of 293T cells with MβCD

(Fig.6A), suggesting that CXCR4 is partially transferred to non–raft domains from raft domains by the MβCD treatment in 293T cells.   However, the transfer of CXCR4 to non-raft domains by the MβCD treatment in NP2 and TE671 cells was not observed, because majority of CXCR4 molecules were originally localized to the non-raft domains in the cells.

To further assess the localization of CXCR4 in the plasma membrane, confocal laser scanning microscopy using the anti-CXCR4 antibody and CT-B was performed.   In almost all 293T cells examined, CXCR4 was co-localized with CT-B, indicating that CXCR4 is largely localized to the raft domains in the MβCD-untreated 293T cells (Fig.6B, the most upper panels).   However, the fluorescent signals of the CXCR4 and CT-B were not matched in about 5 % of MβCD-treated 293T cells (Fig.6B, the second panels).   The data indicate that CXCR4 is partially transferred to non-raft domains by the MβCD treatment.

In contrast to the 293T cells, the individual dot signals of CXCR4 were not completely matched to those of CT-B in the almost all TE671 cells (Fig.6B, third panel, and arrows in the bottom panel).   Interestingly, when two or more cells came in contact, CXCR4 molecules at cell-cell adhesion site appeared to be colocalized with CT-B (Fig. 6B bottom panel).   The result may imply clustering of CXCR4 into raft domains at the cell-cell contact site, and is compatible with previous observations that contact between HIV-1 Env-expressing and receptor-expressing cells can induce translocation of CXCR4 from non-raft to raft regions (Manes et al., 2000; Nguyen et al., 2005), although the CXCR4 localization to raft domains at cell-cell contact site was independent of HIV-1 Env in our study.   Raft localization of CXCR4 in NP2 cells could not be analyzed, because CT-B did not bind to NP2 cells.   These results of cell-staining studies with the 293T and TE671 cells were consistent with the results of cell fractionation studies of Fig.6A.

MβCD inhibits soluble CD4-induced CD4-dependent infection.

The MβCD treatment significantly inhibited the CD4-independent HIV-1 infection, but did not the CD4-dependent infection in the cells exogenously expressing CD4. To know whether the CD4-dependent infection does not require raft microdomains of the target cells, effect of MβCD on soluble CD4 (sCD4)-induced CD4-dependent infection was analyzed. In this experiment, CD4-dependent infection occurs independently of CD4 localization to raft domains. Interestingly, the MβCD treatment significantly inhibited the sCD4-dependent vector (NDK and HXB2) infection (Fig. 7), indicating that sCD4-induced CD4-dependent infection requires the raft membrane domains of the target cells. The result is compatible with the previous report that HIV-1 infection is inhibited by MβCD in cells expressing a CD4 mutant that is localized to non-raft domains (Popik et al., 2004).

Discussion

The raft domains are thought to participate in versatile biological events, such as signal transductions and cell-cell communications, as a scaffold for clustering particular membrane proteins. In this study, we examined potential roles of the raft in the X4-tropic HIV-1 infections. Previous puzzling observations (Del Real et al., 2002, Popik and Alce, 2004; Percherancier et al., 2003) prompted us to examine the possibility that raft localization of CXCR4 rather than CD4 is primarily required for X4-tropic HIV-1 infection. To avoid complications of CD4-dependent infection system, we used CD4-independent or sCD4-induced HIV-1 infection systems.

CD4-independent variants are thought to be prototypes of CD4-dependent variants, and to show fundamental entry pathway shared by both CD4-dependent and –independent viruses (Paolo, 2006; Kubo et al., 2007). Because the CD4-independent and sCD4-induced HIV-1 infections occur independently of CD4 raft localization, they are useful to study the function of CXCR4 raft localization in HIV-1 infection. The MβCD treatment significantly inhibited the CD4-independent (Fig. 1) and sCD4-induced CD4-dependent HIV-1 infections (Fig. 7). In addition, the CXCR4 over-expression did not affect the sensitivity of CD4-independent infection to the MβCD treatment (Fig. 3). These results indicate that raft localization of CXCR4 is required for these HIV-1 infections.

CXCR4 molecules were localized in the raft domains at cell-cell contact regions, but did not at exposed membrane regions (Fig. 6). The HIV-1 vector particles should bind to the CXCR4 molecules in the exposed cell surface regions, in which CXCR4 is localized to non-raft domains. How do the CXCR4 molecules present in non-raft domains function for the X4-tropic HIV-1 infection? It has been already reported

that CXCR4 clusters to raft domains after HIV-1 binding to the cell surface receptors (Manes et al, 2000; Sorice et al., 2001; Del Real et al., 2002; Nguyen et al., 2005).   Therefore, after the CD4-independent virus binds to CXCR4 in non-raft domains, the complexes could move and cluster in the raft domains and induce membrane fusion for the subsequent viral entry.   Alternatively, binding of the CD4-independent HIV-1 to CXCR4 present in the raft domains, but not that in the non-raft domains, could induce productive infection.   However, the latter possibility is unlikely, because CXCR4 in raft domains was detected in the unexposed cell-cell contact sites in TE671 cells, and NP2 and TE671 cells are as susceptible to the CD4-independent virus infection as 293T cells, in which CXCR4 is mainly localized to the raft domains.   However, the CD4-independent infection in 293T cells was suppressed by MβCD as significantly as that in NP2 and TE671 cells.   Because CXCR4 is originally localized to raft domains in 293T cells, the MβCD treatment could inhibit clustering the raft domains containing CXCR4 molecules in 293T cells.   Taken together, clustering of the CXCR4 in raft domains should be important for the HIV-1 infection.

Why does the MβCD have no effect on the CD4-dependent infection in CD4-expressing cells?   The lack of prominent inhibitory effect in our CD4-dependent infection system is compatible with previous study (Viard et al., 2002).   The study showed that MβCD had no significant inhibitory effects on CD4-dependent infection when the cells expressed exogenously abundant amounts of CD4.   One plausible explanation is that CD4 support the CXCR4 clustering in raft domains after the HIV-1 binding.   The interaction of gp120 to CD4 could induce signals to recruit CXCR4 to cluster at the virus-binding site by regulating cytoskeleton dynamics (Iyengar et al., 1998; Viard et al., 2002; Kubo et al., 2008).   It has been reported that the MβCD treatment slightly decreased cholesterol levels of the target cells (Lu et al., 2002), indicating that raft domains still exist in

the MβCD-treated cells.　 Even the partial restriction of raft domains by MβCD should significantly inhibit the CD4-independent infection without the CD4 support, because certain numbers of the receptor molecules are required for the infection.　 Because the sCD4-induced infection was significantly inhibited by MβCD, the cytoplasmic domain-lacking sCD4 could not induce the signals.　 Further studies are needed to clarify this issue.

　 There are many evidences showing that the HIV-1 gp120-CD4-coreceptor complexes are clustered in the raft domains.　 However, it is not clear what is the determinant for clustering the viral complexes in the raft domains.　 It has been reported that the CD4 mutants that do not localize to raft domains can support HIV-1 infection, and the HIV-1 infection through the CD4 mutants is sensitive to the MβCD treatment (Popik, and Alce, 2004), indicating that CD4 is not the determinant.　 It was found in this study that the MβCD treatment significantly inhibits the CD4-independent X4-tropic HIV-1 infection, even when CXCR4 is over-expressed in the target cells, indicating that the raft localization of CXCR4 is absolutely required for the CD4-independent infection.　 In addition, the soluble CD4-induced HIV-1 infection was significantly inhibited by the MβCD treatment.　 These results provide that the raft localization of CXCR4, rather than CD4, is one of the key steps for the HIV-1 entry, and suggest that the interaction between the HIV-1 Env protein and the wild type CD4 supports the clustering of raft domains containing CXCR4.

Acknowledgements

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Figure legends

Fig.1 **Effects of MβCD on HIV-1 infection by different Env protein.**

(A) Effects of MβCD on VSV-G-pseudotyped vector infection in NP2 cells expressing CXCR4 alone (NP2/X4) or both of CXCR4 and CD4 (NP2/CD4/X4).   Cells were treated with 0, 1, 5 mM MβCD for 30 min at 37℃.

(B) Effects of MβCD on CD4-independent mNDK Env-pseudotyped HIV-1 vector infection in NP2/X4 and NP2/CD4/X4 cells.   Cells were treated with 0, 1, 5 mM MβCD for 30 min at 37℃.   (C) Effects of MβCD on the CD4-dependent NDK-pseudotyped vector in NP2/CD4/X4 cells.   (D) Abrogation of MβCD inhibitory effects on mNDK vector infection by cholesterol.   The cells were pre-treated for 30 min at 37℃ as indicated. Relative transduction titers to those in untreated cells are indicated.   This experiment was repeated three times and results are shown as means +SD.   Asterisks indicate statistically significant differences compared to their controls.   (E) Cholesterol levels in HeLa/CD4 or TE671/CD4 cells after MβCD treatment.   MβCD-treated cells were stained with filipin, and fluorescence strength at 525 nm was analyzed by a flow cytometer.   Closed area indicates cells unstained with filipin as a negative control.   Open area indicates MβCD-untreated and filipin-stained cells.   Red lines indicate MβCD-treated and filipin-stained cells.

Fig.2 **Effect of CXCR4 overexpression on mNDK HIV-1 vector infection.**

(A) Cell-surface expression of CXCR4 in original NP2, 293T, and TE671 cells and their CXCR4-overexpressing cells was analyzed by a flow cytometer using the anti-CXCR4 antibody (A80).   Closed and open areas indicate the cells that were incubated in the absence and presence of the A80 antibody, respectively.   (B) Relative transduction titers of the VSV-G (open bar) and mNDK (closed bar) vectors in the CXCR4-overexpressing cells

to those in the original cells are indicated.   This experiment was repeated three times and results are shown as

means +SD.   Asterisks indicate statistically significant differences compared to their controls.

Fig.3 **Effects of MβCD on mNDK HIV-1 vector infection in CXCR4-overexpressing TE671 and 293T cells.**

Relative transduction titers of the VSV-G (open bar) and mNDK (closed bar) vectors in MβCD-treated,

CXCR4-overexpressing TE671 and 293T cells to those in untreated cells are indicated.   Cells were treated with

0, 1, 5 mM MβCD for 30 min at 37℃.   Relative transduction titers to those in untreated cells are indicated.

This experiment was repeated three times and results are shown as means +SD.   Asterisks indicate statistically

significant differences compared to their controls.

Fig.4 **Effect of MβCD on HIV-1 Env-mediated fusion.**

HEK293T effector cells were transfected with the tat or mNDK Env expression plasmid.   The mNDK Env

expression plasmid is designated as tat-Env, because it encodes the Tat protein as well as the Env protein. The

target cells were transfected with the plasmids and treated by MβCD alone or both of MβCD and cholesterol as

indicated in upper panel.   These cells were mixed and LacZ activities of the cell lysates were measured as

shown in Materials and Methods.   Relative values to LacZ activity of untreated cells were indicated.   Asterisks

indicate statistically significant differences compared to their controls.

Fig.5 **Effect of MβCD on CXCR4 expression.**

Cell-surface expression of CXCR4 in NP2/X4, 293T, and TE671 cells was analyzed by a flow cytometer.

Upper panels indicate CXCR4 expression in untreated cells as control, and bottom panels indicate CXCR4 expression in MβCD-treated cells.   Closed and open areas indicate cells that were incubated in absence and presence of the A80 antibody, respectively.

Fig. 6 **Raft localization of CD4 and CXCR4 proteins in the different cells**.

(A) Cell lysates were prepared from MβCD-treated and untreated cells with 0.1% Triton X-100, and the soluble and insoluble fractions are defined as non-raft and raft domains, respectively as shown in Materials and Methods. Cell lysates were electrophoresed on 7.5% polyacrylamide gels and Western blotting was performed using anti-CD4 and anti-HA antibodies.   Molecular size standards are indicated on the left side of the panel.   Arrows indicate CXCR4 molecules.   (B) CXCR4 expressing cells were cultured on four-well culture slides for 24 hr. MβCD-untreated (first panel) and treated (second panel) 293T/X4, and untreated TE671/X4 (third and forth panels) cells were incubated with the rat anti-CXCR4 antibody for 1 hr at 4℃, followed by AlexaFlour 555-conjugated CT-B (red) and FITC-conjugated anti-rat IgG (green) for 1 hr at 4℃.   Representative results are shown.   The colocalization of red and green gives yellow staining.

Fig. 7 **Effect of MβCD on soluble CD4-induced CD4-dependent HIV-1 infection.**

(A) The HIV-1 vector having the NDK Env protein was inoculated into indicated cells in presence of soluble CD4 (sCD4) (20 μg/ml).   (B) The HIV-1 vector having the HXB2 Env protein was inoculated into treated TE671 cells in presence of sCD4.   Relative transduction titers to those in untreated cells are indicated.   These experiments were independently repeated three times.   Asterisks indicate statistically significant differences

compared to their controls.

Fig.1

Fig.2

Fig.3

Fig.4

lane	effector cell	target cell
tat	tat	LTR-LacZ
untreated	tat-Env	LTR-LacZ
MβCD	tat-Env	LTR-LacZ MβCD
MβCD+chol	tat-Env	LTR-LacZ MβCD+chol

Fig.5

Fig.6

A

Fig.6

B

Fig. 7