diseases), and/or suppress immune responses (such as in organ/tissue allotransplantation). Thus, care needs to be taken in the use of proper sets of DCs and their biological characteristics thoroughly tested in animal models prior to their use in human studies. In particular, there is a need for making sure that utilization of DC-based immunotherapy does not inadvertently initiate an autoimmune response.

Studies are in progress to explore whether the present 4B-DCs are able to induce HIV-1-specific $CD8^+$ and $CD4^+$ T cell responses *in vitro* and *in vivo* using our hu-PBL-SCID mouse model (9).

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

Haruka Kamiyama [a,b], Hiroaki Yoshii [a], Yuetsu Tanaka [c], Hironori Sato [a,d], Naoki Yamamoto [a,e], Yoshinao Kubo [a,*]

[a] Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, Nagasaki 852-8523, Japan
[b] Department of Cellular Biochemistry, Graduate School of Human Health Science, Siebold University of Nagasaki, Nagayo, Nagasaki 851-2195, Japan
[c] Department of Immunology, Graduate School and Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan
[d] Laboratory of Viral Genomics, Center for Pathogen Genomics, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama-shi, Tokyo 208-0011, Japan
[e] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

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Human immunode ciency 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- -cyclodextrin (M CD), to address the requirement of raft domain in the X4-tropic virus infection. Treatment of CD4-negative, CXCR4-positive human cells with M CD 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 M CD. The M CD had no effect on the levels of cell surface expression of CXCR4. In contrast to these infections, M CD 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.

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Introduction

Human immunode ciency 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 ef cient 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 evidence 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

* Corresponding author. Fax: +81 95 819 7805.
E-mail address: yoshinao@net.nagasaki-u.ac.jp (Y. Kubo).

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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 micro-domains, 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- -cyclodextrin (M CD) (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.

Results

M CD 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 M CD (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 M CD did not suppress but rather increased the VSV-G-pseudotyped virus infection (Fig. 1A). This M CD 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 M CD treatments of the CD4-negative, CXCR4-positive cells (Fig. 1B, gray bars, NP2/X4 cells). In contrast, inhibitory effects of M CD 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 5 mM of M CD (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 signi cantly inhibited by M CD (Fig. 1C). These results show that M CD inhibits CXCR4-mediated infection but co-expression of CD4 counteracts the inhibitory effects. When excess of cholesterol was added, the inhibitory effect of M CD on the CD4-independent infection was abrogated (Fig. 1D), con rming that cholesterol extraction is a primary cause of suppression of the CD4-independent infection by M CD.

To assess if the M CD treatment indeed had depleted cholesterol from target cell membranes, cells were stained with the cholesterol-binding agent, lipin. Binding of lipin to cholesterol decreases lipin uorescence at 525 nm (Severs and Robenek, 1983; Castanho et al., 1992). Fluorescence strength of lipin in the M CD-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 M CD treatment in our experimental system.

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

In our CD4-independent infection system, virus enters into cells using endogeneously expressed CXCR4. To know whether M CD 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 ow 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 M CD was analyzed. The M CD 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 M CD 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 M CD alone or M CD plus cholesterol occasionally conferred transduction titers of the VSV-G and mNDK vectors higher (Figs. 1A, D, and 3). The mechanism was not understood.

M CD treatment inhibits CD4-independent HIV-1 Env-mediated cell-cell fusion

The above result showed that the depletion of cholesterol by M CD 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 M CD. LacZ activities of the cells were comparable between the cells with and without

the M CD treatment (data not shown), indicating that the M CD treatment does not affect LacZ functional expression. In NP2/CD4/X4 cells, the mNDK Env-mediated cell-cell fusion was not signi cantly inhibited by M CD (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 293 T). The results are consistent with previous study (Ablan et al., 2006). The cell–cell fusion activity inhibited by M CD was recovered, when excess of cholesterol was added into the culture (Fig. 4, M CD+chol). The results were compatible with those of cell-free virus infection (Figs. 1 and 3). Taken together, our results suggest that cholesterol in the cell membrane played an important role in the CD4-independent HIV-1 Env-induced cell–cell fusion and virus entry.

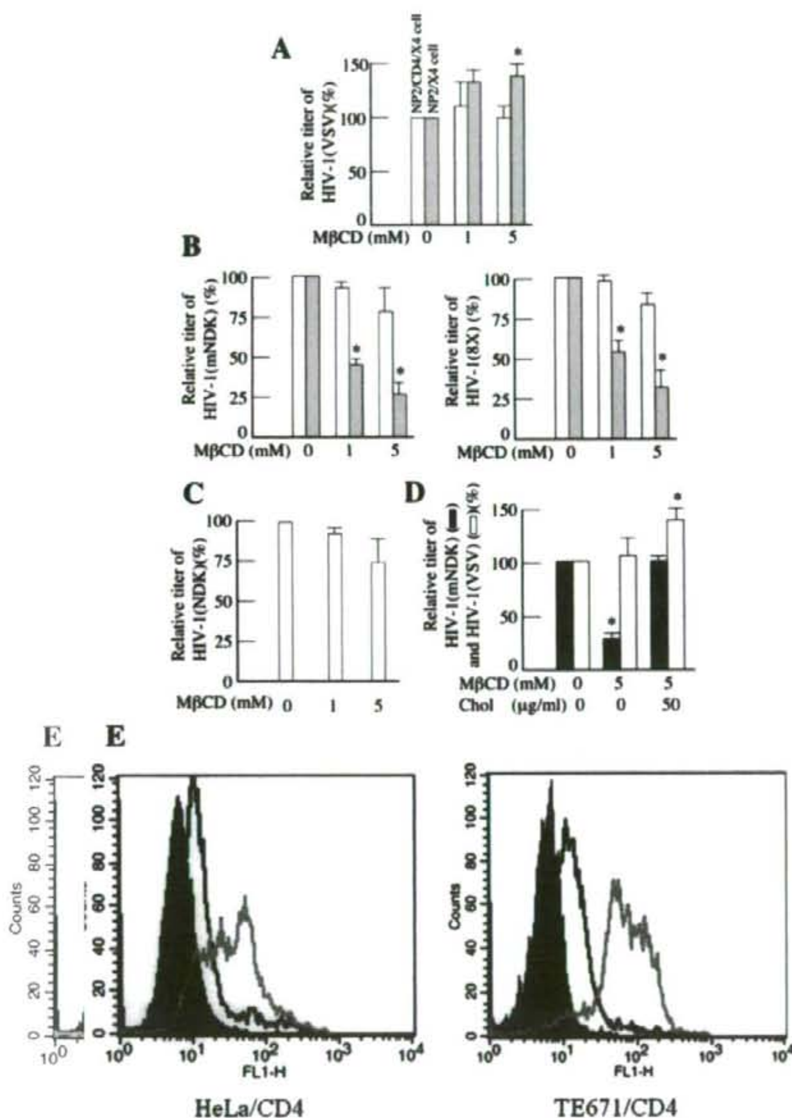


Fig. 1. Effects of M CD on HIV-1 infection by different Env protein. (A) Effects of M CD on VSV-G-pseudotyped vector infection in NP2 cells expressing CXCR4 alone (NP2/X4) or both of CXCR4 and CD4 (NP2/CD4/X4). Cells were treated with 0, 1, 5 mM M CD for 30 min at 37 °C. (B) Effects of M CD on CD4-independent mNDK Env-pseudotyped HIV-1 vector infection in NP2/X4 and NP2/CD4/X4 cells. Cells were treated with 0, 1, 5 mM M CD for 30 min at 37 °C. (C) Effects of M CD on the CD4-dependent NDK-pseudotyped vector in NP2/CD4/X4 cells. (D) Abrogation of M CD inhibitory effects on mNDK vector infection by cholesterol. The cells were pre-treated for 30 min at 37 °C 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 signi cant differences compared to their controls. (E) Cholesterol levels in HeLa/CD4 or TE671/CD4 cells after M CD treatment. M CD-treated cells were stained with lipin, and uorescence strength at 525 nm was analyzed by a ow cytometer. Closed area indicates cells unstained with lipin as a negative control. Open area indicates M CD-untreated and lipin-stained cells. Gray lines indicate M CD-treated and lipin-stained cells.

To assess the possibility that M CD altered the CXCR4 expression, expression levels of CXCR4 in the treated cells were analyzed by a ow cytometer. Treatment of M CD did not affect the expression of CXCR4 in NP2/X4, 293 T, and TE671 cells (Fig. 5). This result indicates that the inhibition of CD4-independent infection by M CD 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 de ned as the raft membrane domains (Simons and Ikonen, 1997). Using this approach, we examined localization of CXCR4 in the raft domains in the presence or absence of M CD. 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 and 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 M CD (Fig. 6A), suggesting that CXCR4 is partially transferred to non-raft domains from raft domains by the M CD treatment in 293T cells. However, the transfer of CXCR4 to non-raft domains by the M CD 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 M CD-untreated 293T cells (Fig. 6B, the most upper panels). However, the uorescent signals of the CXCR4 and CT-B were not matched in about 5% of M CD-treated 293 T cells (Fig. 6B, the second panels). The data indicate that CXCR4 is partially transferred to non-raft domains by the M CD treatment.

In contrast to the 293 T 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

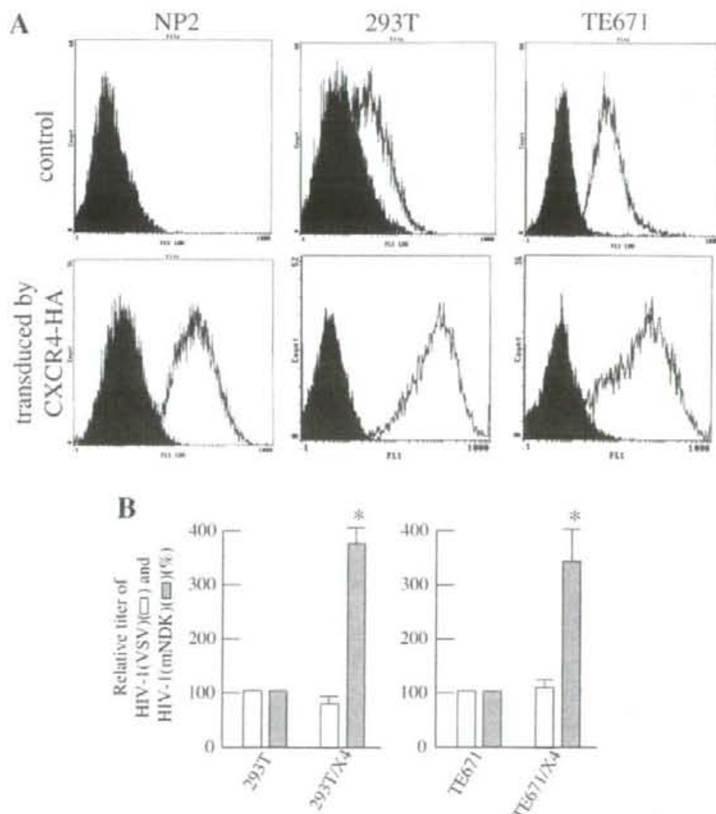


Fig. 2. Effect of CXCR4 overexpression on mNDK HIV-1 vector infection. (A) Cell-surface expression of CXCR4 in original NP2, 293 T, and TE671 cells and their CXCR4-overexpressing cells was analyzed by a ow cytometer using the anti-CXCR4 antibody (A80). Closed and open areas indicate the cells that were 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 signi cant differences compared to their controls.

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 293 T and TE671 cells were consistent with the results of cell fractionation studies of Fig. 6A.

M CD inhibits soluble CD4-induced CD4-dependent infection

The M CD treatment signi cantly 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 M CD 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 M CD treatment signi cantly 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 M CD in cells expressing a CD4 mutant that is localized to non-raft domains (Popik and Alce, 2004).

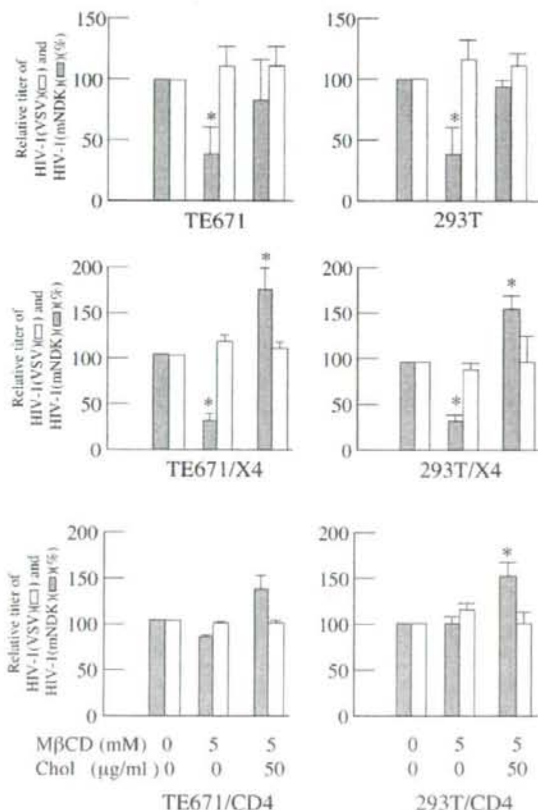


Fig. 3. Effects of M CD on mNDK HIV-1 vector infection in CXCR4-overexpressing TE671 and 293T cells. Relative transduction titers of the VSV-G (open bar) and mNDK (closed bar) vectors in M CD-treated, CXCR4-overexpressing TE671 and 293 T cells to those in untreated cells are indicated. Cells were treated with 0, 1, 5 mM M CD for 30 min at 37 °C. 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 signi cant differences compared to their controls.

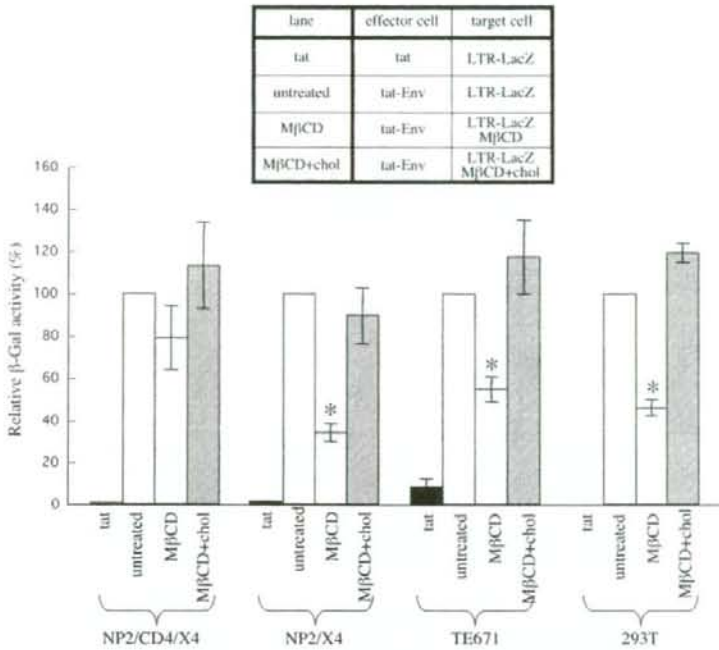
Discussion

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 M CD treatment signi cantly 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 M CD 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 M CD as signi cantly as that in NP2 and TE671 cells. Because CXCR4 is originally localized to raft domains in 293T cells, the M CD 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 M CD 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 M CD had no signi cant 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 M CD treatment slightly decreased cholesterol levels of the target cells (Lu



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. 4. Effect of M CD 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 M CD alone or both of M CD 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 signi cant differences compared to their controls.

et al., 2002), indicating that raft domains still exist in the M CD-treated cells. Even the partial restriction of raft domains by M CD should signi cantly 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 signi cantly inhibited by M CD, the cytoplasmic domain-lacking sCD4 could not induce the signals. Further studies are needed to clarify this issue.

There are many evidence showing that the HIV-1 gp120–CD4-coreceptor complexes are clustered in the raft domains. However,

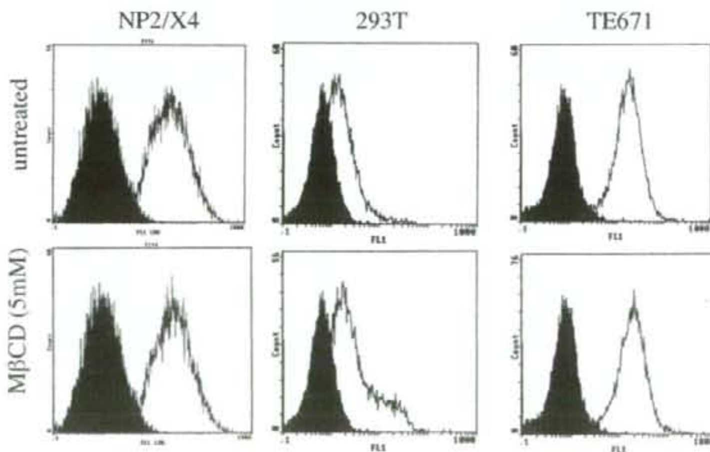


Fig. 5. Effect of M CD on CXCR4 expression. Cell-surface expression of CXCR4 in NP2/X4, 293 T, and TE671 cells was analyzed by a ow cytometer. Upper panels indicate CXCR4 expression in untreated cells as control, and bottom panels indicate CXCR4 expression in M CD-treated cells. Closed and open areas indicate cells that were incubated in absence and presence of the A80 antibody, respectively.

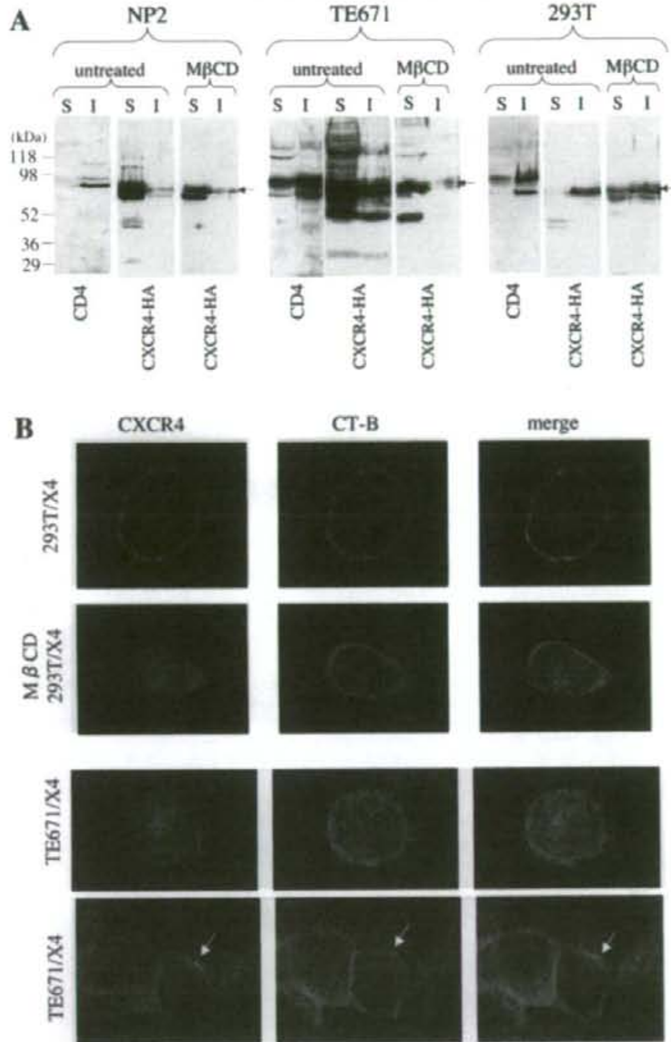


Fig. 6. Raft localization of CD4 and CXCR4 proteins in the different cells. (A) Cell lysates were prepared from M CD-treated and untreated cells with 0.1% Triton X-100, and the soluble and insoluble fractions are de ned as non-raft and raft domains, respectively as shown in Materials and methods. Cell 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 h . M CD-untreated (rst 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 h at 4 °C, followed by AlexaFlour 555-conjugated CT-B (red) and FITC-conjugated anti-rat IgG (green) for 1 h at 4 °C. Representative results are shown. The colocalization of red and green gives yellow staining.

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 M CD treatment (Popik and Alce, 2004), indicating that CD4 is not the determinant. It was found in this study that the M CD treatment signi cantly 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 signi cantly inhibited by the M CD 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.

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

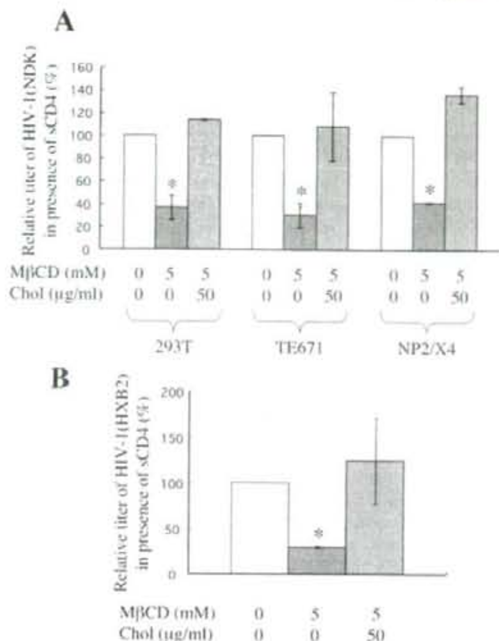


Fig. 7. Effect of M CD on soluble CD4-induced CD4-dependent HIV-1 infection. (A) The HIV-1 vector having the NDK Env protein was inoculated into indicated cells in presence of soluble CD4 (sCD4) (20 g/ml). (B) The HIV-1 vector having the HXB2 Env protein was inoculated into treated TE671 cells in presence of sCD4. Relative transduction titers to those in untreated cells are indicated. These experiments were independently repeated three times. Asterisks indicate statistically signi cant differences compared to their controls.

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) 293 T cell were cultured in Dulbecco's modi ed Eagle's medium (Sigma) at 37 °C in 5% CO_2. The culture media were supplemented with 8% fetal bovine serum (Bio uids). 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 (3 g) (TAKARA), CD4-encoding retroviral vector (3 g), and VSV-G expression plasmid (3 g) (Chang et al., 1999) by the TransIT LT1 regent (30 l) (Mirus). The cells were washed 24 h after transfection, and cultured for 24 h in fresh medium. Culture supernatant of the transfected cells was inoculated into 293 T or TE671 cells. The inoculated cells were selected by puromycin (10 g/ml). The puromycin-resistant cell pool was utilized in this study. TE671 and 293 T 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) (Naldini et al., 1996), LacZ-containing HIV-1 vector (Iwakuma et al., 1999), and the appropriate Env expression plasmids (3 g each) by the Trans IT-LT1 reagent. The transfected cells were washed with medium 24 h after transfection, and continued to be cultured in fresh medium for 24 h . Target cells were inoculated with the culture supernatants of the transfected cells in presence or absence of soluble CD4 (20 g/ml) (obtained from AIDS Research and Reference Reagent program, NIH). The inoculated cells were stained with 5-bromo-4-chloro-3-indolyl- -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- ¯cyclodextrin (M CD) (Sigma) in FBS-free medium, or with 5 mM M CD and 50 g/ml cholesterol (Wako) for 30–120 min at 37 °C. 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 M CD and cholesterol.

Filipin staining

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

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To analyze cell surface expression of CXCR4, suspended cells were treated with a rat anti-CXCR4 antibody (A80) for 1 h at 4 °C. 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 ow 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 M CD. These cells were mixed 48 h after transfection, and cultured for 24 h . LacZ activities of the cell lysates prepared from the mixed cultures were measured by the high sensitive -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 de ned as soluble fraction. Equal volume of sample buffer was added to its precipitates, and it was de ned 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).

Immuno uorescence microscopy

CXCR4 expressing cells were cultured on four-well culture slides (Miles) for 24 h . Cells were incubated with the rat anti-CXCR4 antibody (A80) for 1 h at 4 °C, followed by AlexaFlour 555-conjugated CT-B and FITC-conjugated anti-rat IgG for 1 h at 4 °C. Cells were observed using a confocal uorescence microscope (Leica).

Statistical analysis

Differences between groups of data were deter,imed by Student's t-test. Statistical signi cance was set as P b 0.01 for all tests.

Acknowledgments

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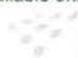
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Ezrin, Radixin, and Moesin (ERM) proteins function as pleiotropic regulators of human immunodeficiency virus type 1 infection

Yoshinao Kubo [a,*], Hiroaki Yoshii [a], Haruka Kamiyama [a], Chika Tominaga [a], Yuetsu Tanaka [b], Hironori Sato [a,c], Naoki Yamamoto [a,d]

[a] Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, Nagasaki, National Institute of Infectious Diseases, Tokyo, Japan
[b] Department of Immunology, University of the Ryukyus, Okinawa, National Institute of Infectious Diseases, Tokyo, Japan
[c] Laboratory of Viral Genomics, Center for Pathogen Genomics, National Institute of Infectious Diseases, Tokyo, Japan
[d] AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

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Abstract

Ezrin, radixin, and moesin (ERM) proteins supply functional linkage between integral membrane proteins and cytoskeleton in mammalian cells to regulate membrane protein dynamisms and cytoskeleton rearrangement. To assess potential role of the ERM proteins in HIV-1 lifecycle, we examined if suppression of ERM function in human cells expressing HIV-1 infection receptors influences HIV-1 envelope (Env)-mediated HIV-1-vector transduction and cell–cell fusion. Expression of an ezrin dominant negative mutant or knockdown of ezrin, radixin, or moesin with siRNA uniformly decreased transduction titers of HIV-1 vectors having X4-tropic Env. In contrast, transduction titers of R5-tropic Env HIV-1 vectors were decreased only by radixin knockdown: ezrin knockdown had no detectable effects and moesin knockdown rather increased transduction titer. Each of the ERM suppressions had no detectable effects on cell surface expression of CD4, CCR5, and CXCR4 or VSV-Env-mediated HIV-1 vector transductions. Finally, the individual knockdown of ERM mRNAs uniformly decreased efficiency of cell–cell fusion mediated by X4- or R5-tropic Env and HIV-1 infection receptors. These results suggest that (i) the ERM proteins function as positive regulators of infection by X4-tropic HIV-1, (ii) moesin additionally functions as a negative regulator of R5-tropic HIV-1 virus infection at the early step(s) after the membrane fusion, and (iii) receptor protein dynamisms are regulated differently in R5- and X4-tropic HIV-1 infections.
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Keywords: HIV-1; Ezrin; Radixin; Moesin

Introduction

Human immunodeficiency virus type 1 (HIV-1) enters into host cells by fusion between viral envelope and host cell membrane following the binding of HIV-1 envelope glycoprotein (Env) to the cell surface receptors, CD4 and coreceptor (CXCR4 or CCR5). The HIV-1 Env glycoprotein is synthesized as a precursor polyprotein, and cleaved to surface (SU) and transmembrane (TM) subunits by a cellular protease. The conformational change of SU subunit by its interaction with CD4 triggers the formation and exposure of the coreceptor binding domain. The binding of SU subunit to the coreceptor molecule activates the membrane fusion capability of the viral TM subunit required for the HIV-1 entry into host cells.

After the HIV-1 Env protein binds to host cells, the HIV-1 infection receptors are clustered (Jolly and Sattentau, 2005; Nguyen et al., 2005; Viard et al., 2002). The receptor clustering requires cytoskeletal functions (Iyengar et al., 1998; Jolly et al., 2004; Kizhatil and Albritton, 1997; Lehmann et al., 2005; Pontow et al., 2004; Steffens and Hope, 2003). This results in multiple interactions between the viral Env proteins and the host receptor molecules on the interacting site between host cell and virion (Jimenez-Baranda et al., 2007; Platt et al., 1998). Although accumulating evidence indicates that the cytoskeleton-dependent clustering of infection receptors are essential for

* Corresponding author. Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, Japan 852-8523. Fax: +81 95 849 7805.

E-mail address: yoshinao@net.nagasaki-u.ac.jp (Y. Kubo).

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efficient membrane fusion and subsequent entry of HIV-1 into the target cells, there is no evidence that the receptor proteins, CD4, CXCR4, and CCR5, directly bind to the cytoskeleton. This suggests that some linker molecules between the receptor and cytoskeleton is involved in the HIV-1 entry.

Ezrin, radixin, and moesin are the cytosolic proteins called ERM family that supplies functional linkage between integral membrane proteins and cytoskeleton (Algrain et al., 1993; Fievet et al., 2006; Tsukita et al., 1997). They are highly homologous each other, sharing about 70–80% amino acid identity. The ERM plays key roles in cell morphogenesis and communication via regulating membrane protein dynamisms and cytoskeleton rearrangement (Tsukita et al., 1997). The ERM, particularly ezrin, is important for reconstructing cell-surface architecture during T cell activation (Das et al., 2002; Faure et al., 2004; Gupta et al., 2006; Roumier et al., 2001). Despite the general similarity in structure and function, individual function of the three proteins appears to be specialized (Doi et al., 1999; Kikuchi et al., 2002; Saotome et al., 2004; Takeuchi et al., 1994). Ezrin is crucial for formation of local architecture, called immunological synapse, at the contact site of antigen-presenting and T cells (Gupta et al., 2006; Roumier et al., 2001). Dephosphorylation and relocation of

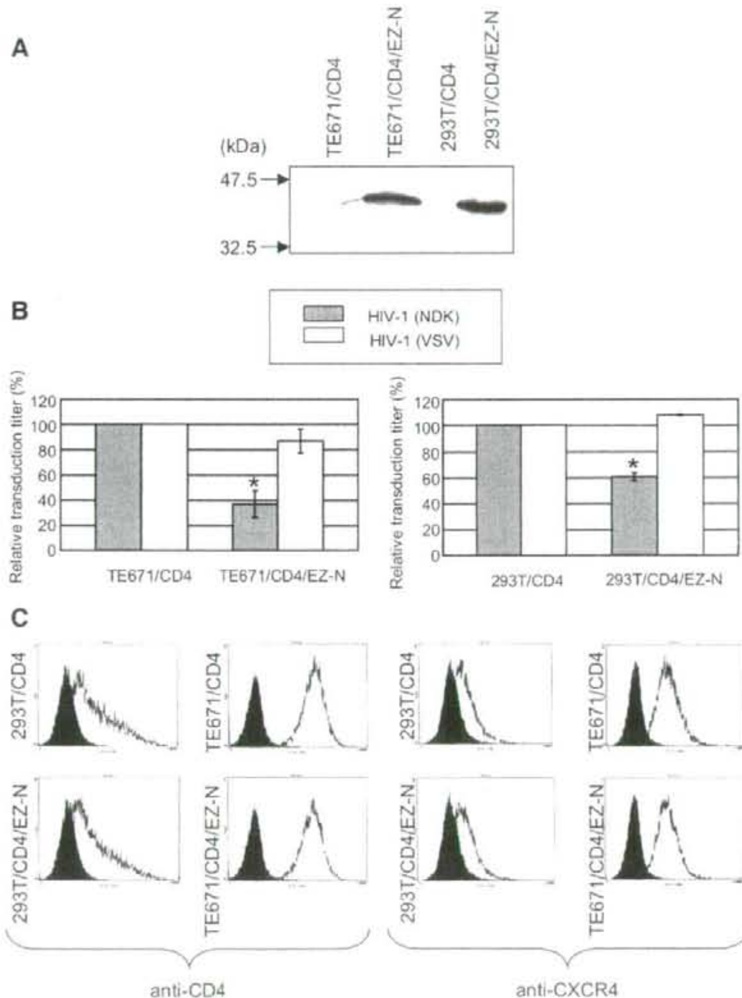

Fig. 1. Effect of ezrin dominant negative mutant on vector transduction. Panel A. Cell lysates prepared from cells expressing the VSV-G epitope-tagged ezrin dominant negative mutant (EZ-N) were subjected to Western immunoblotting using the anti-VSV-G epitope antibody. Molecular size markers are indicated in left side of the panel. Panel B. Transduction titers of the HIV-1 vector having VSV-G (open bar) or NDK HIV-1 Env (closed bar) protein were measured in the control and EZ-N-expressing cells. Relative values to the titer in the control cells are shown. Three independent experiments were performed. Error bars indicate standard deviations. Panel C. Cell surface expression of CD4 and CXCR4 in the control and the EZ-N-expressing cells was analysed by FACS. Closed areas indicate cells stained without the first antibody. Open areas indicate cells stained with the anti-CD4 or anti-CXCR4 antibody.

ezrin trigger transient uncoupling of lipid rafts or plasma membrane from the actin cytoskeleton, which presumably increases lipid raft dynamics and T cell receptor clustering (Gupta et al., 2006). The immunological synapse contains CD4 and CXCR4 (Roumier et al., 2001), the HIV-1 infection receptors, and its formation requires cytoskeleton rearrangement (Das et al., 2002). In addition, the HIV-1 receptors have been reported to co-localize with ezrin (Steffens and Hope, 2003).

In this study, we examined potential roles of the ERM proteins in the HIV-1 infection. We prepared HIV-1-susceptible human cells in which function or expression of the ERM family proteins were suppressed by either ezrin dominant negative mutant (Algrain et al., 1993) or by RNA interference technique (Rana, 2007). Suppression of individual ERM in the target cells yielded distinct effects on HIV-1 vector transductions mediated by the X4- and R5-tropic Env proteins. The ERM suppressions uniformly inhibited cell–cell fusion mediated by the X4- and R5-tropic envelope proteins. Our findings provide for the first time the evidence that the ERM family proteins function as pleiotropic regulators of HIV-1 infection.

Results

Ezrin dominant negative mutant inhibits X4-tropic HIV-1 vector infection

The N-terminal and C-terminal domains of ezrin bind membrane proteins and cytoskeleton, respectively (Tsukita et al., 1997). Expression of an ezrin N-terminal domain has been reported to interfere with the endogenous ezrin function as a dominant negative mutant (Algrain et al., 1993; Roumier et al., 2001). To examine if the ezrin influences HIV-1 infection, we prepared human cells expressing the ezrin dominant negative mutant (EZ-N) that is C-terminally tagged with the VSV-G epitope (Algrain et al., 1993). A murine leukemia virus (MLV) vector carrying the EZ-N was inoculated into 293T and TE671 cells expressing CD4 (293T/CD4 and TE671/CD4), and the cells were selected by puromycin. Because the MLV vector genome contained both of the EZ-N and puromycin-resistant genes, it was thought that almost all of the puromycin-resistant cells expressed the EZ-N protein. Western immunoblotting using the anti-VSV-G epitope antibody indicates that the puromycin-resistant cell pools expressed the VSV-G-tagged EZ-N protein as the predicted size (Fig. 1A).

The parental TE671/CD4 and 293T/CD4 cells were susceptible to HIV-1 vector having the X4-tropic NDK HIV-1 Env protein, because the cells endogenously express CXCR4. Transduction titers of the HIV-1 NDK Env vector in TE671/CD4 and 293T/CD4 cells expressing the EZ-N were constantly decreased (Fig. 1B). Such reduction in virus titer was observed when HIV-1 vector having Env protein of a CD4-independent X4-tropic virus strain, mNDK (Dumonceaux et al., 1998), was used as a transduction source (data not shown). In contrast, the EZ-N expression had little effects on the infection of HIV-1 vector having VSV-G protein. Expression of the EZ-N had little effects on cell surface expression of the HIV-1 receptors, CD4 and CXCR4, as monitored by FACS analysis (Fig. 1C). These results suggest that ezrin functions as a positive regulator of HIV-1 infection mediated by the X4-tropic HIV-1 Env but not by the VSV-Env.

To assess whether the EZ-N affects R5-tropic HIV-1 vector transduction, transduction titers of the HIV-1 vector having the R5-tropic JRFL HIV-1 Env protein were measured in target cells transiently transfected with the VSV-G-tagged wild type ezrin (EZ-Wt) or the EZ-N expression plasmid. TE671 cells expressing CD4 and CCR5 (TE671/CD4/R5) were used as the target cells. The EZ-N reduced transduction titer of the X4-tropic NDK HIV-1 vector in the TE671/CD4/R5 cells (Fig. 2A)

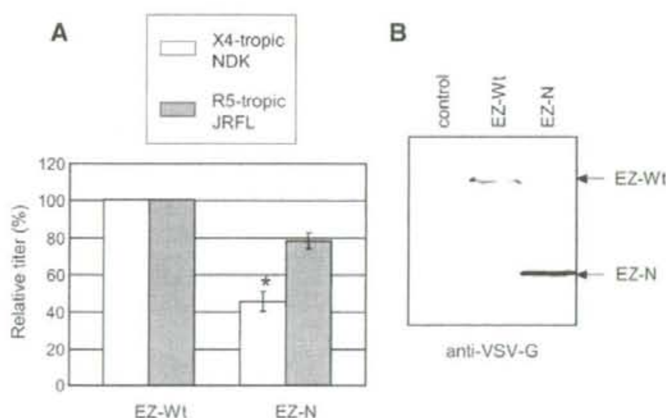

Fig. 2. Effect of ezrin dominant negative mutant on R5-tropic HIV-1 vector transduction. Panel A. Transduction titers of the HIV-1 vector having the X4-tropic NDK (open bar) or R5-tropic JRFL (closed bar) Env protein were measured in TE671/CD4/R5 cells transiently transfected with the wild type ezrin (EZ-Wt) or ezrin dominant negative mutant (EZ-N) expression plasmid. Relative titers to that in the EZ-Wt-transfected cells were indicated. This experiment was repeated three times, and error bars indicate standard deviations. Panel B. Western immunoblotting of the transfected cells was performed using the anti-VSV-G epitope antibody. The EZ-Wt and EZ-N proteins are estimated by their molecular sizes, and are shown by arrows.

compared to the EZ-Wt as control. This result was consistent with that in the TE671/CD4 cells stably expressing the EZ-N (Fig. 1B). However, the EZ-N did not significantly affect the R5-tropic JRFL HIV-1 vector transduction efficiency.

Additionally, we analyzed the effects of the EZ-N on transduction activity and syncytium formation of ecotropic MLV Env proteins in rat XC cells (Kubo et al., 2003). These functions of the ecotropic MLV Env proteins were not affected by the EZ-N

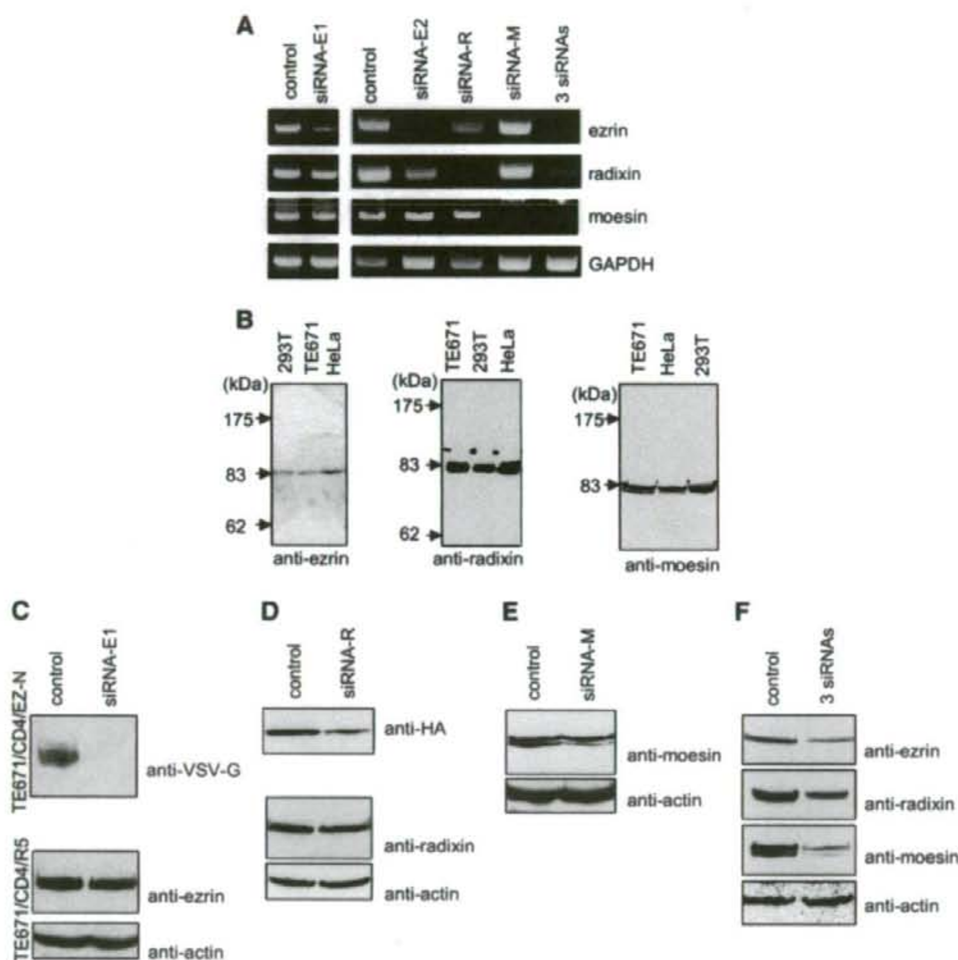

Fig. 3. Knockdown of ERM family proteins by siRNAs. Panel A. Effects of siRNA on ERM mRNA expression. Total RNA samples were isolated from siRNA-GFP-, siRNA-E1-, siRNA-E2-, siRNA-R-, or siRNA-M-transfected TE671/CD4/R5 cells and from cells simultaneously transfected with siRNA-E2, siRNA-R, and siRNA-M (3 siRNAs). Semi-quantitative RT-PCR of these total RNA samples was performed to detect ezrin, radixin, moesin, or GAPDH mRNA. Panel B. ERM family protein expression in human cells. Cell lysates were prepared from 293T, TE671, and HeLa cells, and subjected to Western immunoblotting using the anti-ezrin (left panel), -radixin (middle panel), and -moesin (right panel) antibodies. Molecular size markers are indicated in left side of the panels. Panel C. Effects of siRNA-E1 on ezrin protein expression. Cell lysates were prepared from siRNA-GFP (control)- or siRNA-E1-transfected TE671/CD4 cells expressing the VSV-G-tagged EZ-N mutant. Western immunoblotting of the lysates was performed using the anti-VSV-G epitope antibody. Cell lysates were prepared from siRNA-GFP- or indicated siRNA-E1-transfected TE671/CD4/R5 cells. Western immunoblotting of the lysates was performed using the anti-ezrin or anti-actin antibody. Panel D. Effects of siRNA-R on radixin protein expression. TE671/CD4/R5 cells were transiently transfected with the HA-tagged radixin expression plasmid and an siRNA indicated, and cell lysates were prepared from the transfected cells. Western immunoblotting using the anti-HA antibody was performed. Cell lysates were prepapred from TE671/CD4/R5 cells transfected with the siRNA-GFP or -R. Western immunoblotting using the anti-radixin or anti-actin antibody was performed. Panel E. Effects of siRNA-M on moesin protein expression. Cell lysates were prepared from TE671/CD4/R5 cells transfected with the siRNA-GFP or -M. Western immunoblotting using the anti-moesin or anti-actin antibody was performed. Panel F. Effects of these three siRNAs on ERM protein expression. Cell lysates were prepared from TE671/CD4/R5 cells simultaneously transfected with siRNA-E2, -R, and -R and from cells transfected with siRNA-GFP. Western immunoblotting using anti-ezrin, anti-radixin, anti-moesin, or anti-actin antibody was performed.

expression (data not shown), indicating that ezrin is not associated with the ecotropic MLV Env functions.

Knockdown of ERM family protein expression by siRNA inhibits HIV-1 vector transduction

We were interested in the roles of other ERM family proteins, i.e., radixin and moesin, in HIV-1 infection. siRNAs targeting ezrin, radixin, or moesin mRNA was introduced into the TE671/CD4/R5 cells, and level of the mRNA was monitored by semi-quantitative RT-PCR; siRNA-E1 and -E2 target the ezrin mRNA; siRNA-R targets the radixin mRNA; siRNA-M targets the moesin mRNA. As shown in Fig. 3A, these siRNAs specifically and effectively suppressed expression of corresponding ERM family mRNAs in the TE671/CD4/R5 cells.

We next examined if the siRNAs influence expression levels of the ERM family proteins by Western immunoblotting. As described in manufacture's documents of the antibodies, the commercially available antibodies against the ezrin, radixin, and moesin had strong cross-reactivity due to the high homology within the ERM family proteins. However, we could distinguish moesin from others by the anti-moesin antibody because moesin has smaller molecular size (Fig. 3B, anti-moesin). The moesin was expressed at detectable levels in HeLa and TE671 cells but not in 293T cells. The data with anti-ezrin and anti-radixin antibodies suggest that these proteins were expressed in the three human cells.

To examine siRNA suppression effects on ERM protein expression, we first used anti-VSV-G antibody and examined if exogenous expression of the VSV-G-tagged EZ-N mutant protein in TE671/CD4/EZ-N cells is suppressed by the siRNA against ezrin (siRNA-E1). Target sequence of the siRNA-E1 is located in the N terminal protein-coding region of ezrin mRNA and thus expression of the EZ-N should be suppressed if the siRNA-E1 was functional. Fig. 3C shows that the siRNA-E1 suppressed the expression of the VSV-G-tagged EZ-N mutant protein in TE671/CD4/EZ-N cells, indicating that the siRNA-E1 is functional. As expected, however, we failed to detect the siRNA suppression

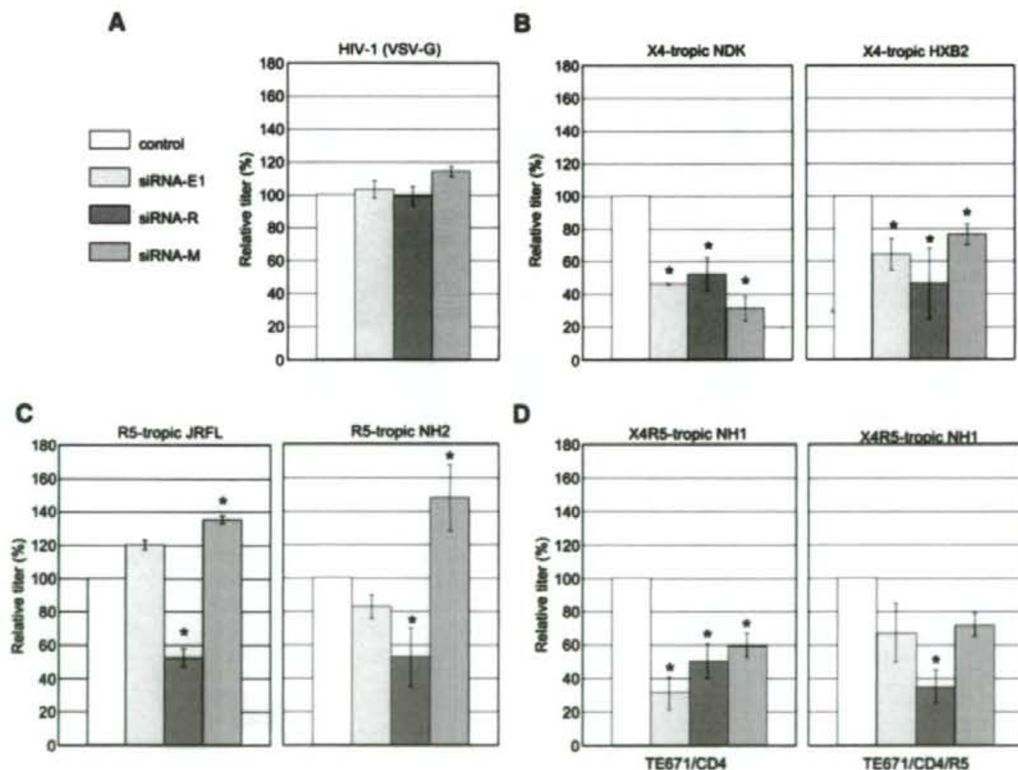

Fig. 4. Effect of siRNAs against ERM family genes on HIV-1 vector transduction. HIV-1 vector pseudotyped with VSV-G (panel A), HIV-1 X4-tropic Env (panel B), or HIV-1 R5-tropic Env (panel C) was inoculated into TE671/CD4/R5 cells transfected with siRNA-GFP, siRNA-E1, siRNA-R, or siRNA-M. HIV-1 vector having the X4R5-tropic NH1 Env protein (panel D) was inoculated into siRNA-transfected TE671/CD4 (left panel) or TE671/CD4/R5 cells (right panel). Relative values to transduction titer in the siRNA-GFP-transfected cells were indicated. This experiment was independently repeated three times. Error bars indicate standard deviations. Asterisks indicate statistical significance (P b 0.05). Panel E. Cell surface expressions of CD4, CXCR4, and CCR5 in the siRNA-transfected TE671/CD4/R5 cells were analyzed by FACS. Closed areas shows cells stained with the FITC-conjugated secondary antibody alone, and open areas do cells stained with the indicated antibodies and the secondary antibody.

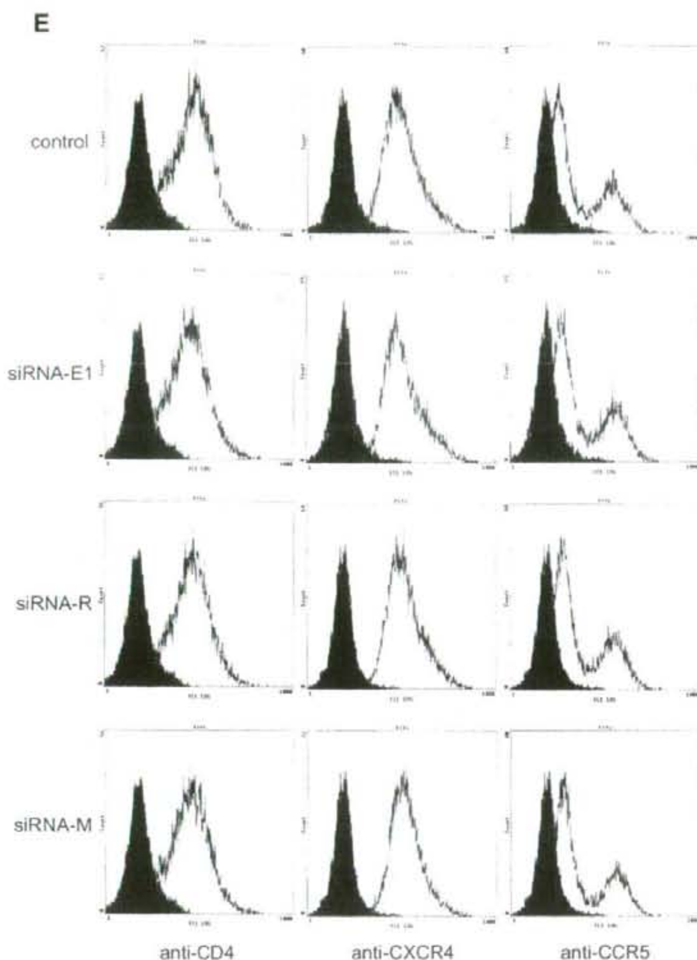

Fig. 4 (continued).

effect in the TE671/CD4/R5 cells with the anti-ezrin antibody (Fig. 3C), because the anti-ezrin antibody recognizes radixin as well. Similarly, we failed to confirm suppression of radixin expression by the siRNA-R with the anti-radixin antibody due to the cross-reactivity of the antibody (Fig. 3D). Therefore, we constructed an expression plasmid of a C-terminally HA-tagged radixin (Rad-HA) to confirm whether the siRNA-R is functional. The Rad-HA level in TE671/CD4/R5 cells co-transfected with the Rad-HA expression plasmid and the siRNA-R was lower than that in cells co-transfected with the Rad-HA expression plasmid and the siRNA against GFP (Fig. 3D). In contrast, we could confirm the siRNA-M-mediated suppression of endogenous moesin protein expression with anti-moesin antibody, because molecular size of moesin is smaller than ezrin and radixin (Fig. 3E). When TE671/CD4/R5 cells were simultaneously transfected with the siRNA-E2, -R, and -M, suppressed expression of ezrin, radixin, and moesin proteins was detected using the each antibodies (Fig. 3F). Taken together, our results suggest that these siRNAs inhibit the corresponding protein expression via suppression of mRNA expression.

Transduction titer of the VSV-G vector was not affected by these siRNAs (Fig. 4A), suggesting that VSV-envelope-mediated infection proceeds via ERM-protein independent pathway as already reported (Kameoka et al., 2007). In contrast, transduction titers of the X4-tropic NDK and HXB2 vectors were decreased uniformly by the introduction of siRNA against ezrin, radixin, or moesin (Fig. 4B). X4-tropic transduction efficiency of the X4R5-tropic NH1 vector was also inhibited in TE671/CD4 cells (Fig. 4D), because CC5R is not expressed in the cells. These results were consistent with the data on the ezrin dominant negative mutant (Figs. 1 and 2).

The siRNA-R decreased the titers of the R5-tropic vector and the siRNA-M rather increased the titers (Fig. 4C). These changes were highly reproducible in the repeated experiments.

These effect of the siRNA-mediated knock down of the ERM proteins on the HIV-1 infection was not induced by altered cell surface expression of the HIV-1 receptors, because cell surface expression of the HIV-1 receptors, CD4, CXCR4, and CCR5, were not changed by the siRNAs (Fig. 4E). These results suggest that all of the three ERM family proteins function as positive regulators of the X4-tropic HIV-1 infection, whereas radixin and moesin function positive and negative regulators, respectively, of the R5-tropic HIV-1 infection.

Transduction efficiency of the dual-tropic NH1 vector in TE671/CD4 cells was suppressed by each of the siRNA (Fig. 4D) as that of the X4-tropic vector. Because TE671/CD4 cells do not express CCR5, entry of the dual-tropic NH1 vector occurs only through CXCR4 in the cells. The moesin knockdown in TE671/CD4/R5 cells did not enhance transduction efficiency of the dual-tropic NH1 vector, but did that of the R5-tropic vector. Entry of the NH1 vector was thought to occur through both of CXCR4 and CCR5 in TE671/CD4/R5 cells. Therefore, the effect of moesin knockdown on the dual-tropic vector in TE671/CD4/R5 cells should be different from that on the R5-tropic vector.

To examine if expression of siRNA-resistant ezrin mRNA abrogate the inhibitory effect of ezrin siRNA on the X4-tropic HIV-1 vector transduction, we examined effects of siRNA-E2, which targets 3 untranslated region (3 UTR) of the ezrin mRNA, on the X4-tropic virus transduction in the TE671/CD4 cells. The siRNA-E2 reduced the endogenous ezrin mRNA level (Fig. 3A), but did not suppress exogenous expression of VSV-G-tagged wild type ezrin (Fig. 5A), because the exogenous mRNA encoding the VSV-G-tagged ezrin does not contain the 3 UTR. The siRNA-E2 transfection into TE671/CD4 cells decreased transduction titer of the NDK HIV-1 vector (Fig. 5B) as the siRNA-E1 did (Fig. 4B). Expression of the siRNA-resistant ezrin, i.e., VSV-G-tagged ezrin wild type protein, abrogated the inhibitory effect of the siRNA-E2 (Fig. 5B). The VSV-G-tagged ezrin expression alone did not affect the HIV-1 vector transduction efficiency. These results support the argument that ezrin is important for increasing efficiency of the X4-tropic HIV-1 infection.

Effects of ERM-family-targeting siRNAs on cell–cell fusion mediated by HIV-1 Env proteins

To assess whether the ERM family proteins play roles in HIV-1-Env-mediated membrane fusion, we examined if the ezrin dominant negative mutant (EZ-N) and siRNAs against the ERM family proteins influence cell–cell fusion in co-culture of target cells and NDK Env-expressing 293T cells. In this co-culture system, we can monitor cell–cell fusion via interaction of HIV-1 Env and HIV-1 infection receptors by using the -galactosidase activity (see Materials and methods). NDK Env-mediated cell–cell fusion was inhibited by introduction of either the EZ-N protein (Fig. 6A), siRNA-E2, -R, or -M (Fig. 6B) into the receptor expressing cells, consistent with the results obtained from HIV-1 vector transduction assay (Figs. 1B and 4B). Similarly, JRFL-Env-mediated cell–cell fusion was inhibited by ezrin and radixin siRNAs (Fig. 6C), although the ezrin siRNA had no effect on the R5-tropic HIV-1 vector transduction efficiency (Fig. 4C). The siRNA-M enhanced the vector transduction of the R5-tropic vector, whereas such enhancement was not observed in the cell fusion. These effects were highly reproducible in the repeated experiments.

Discussion

In this study, we examined potential roles of the ERM proteins in HIV-1 entry. A recent study described the similar topic, in

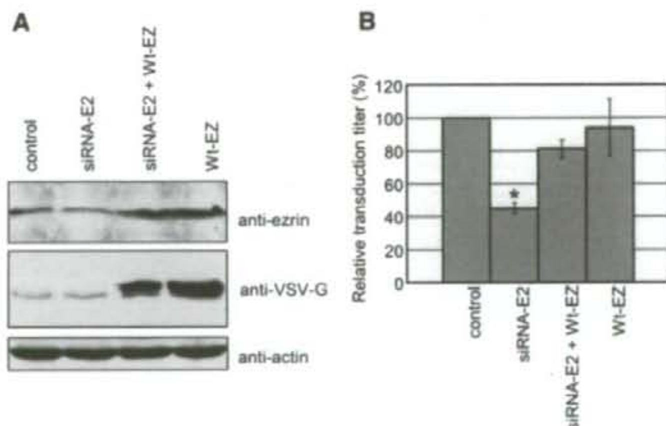

Fig. 5. Abrogation of inhibitory effect of ezrin knockdown on HIV-1 vector transduction by exogenous ezrin expression. Panel A. Cell lystaes were prepared from TE671/CD4/R5 cells transfected with the siRNA-GFP alone, from cells transfected with siRNA-E2 alone, from cells co-transfected with the VSV-G-tagged wild type ezrin expression plasmid and the siRNA-E2, and from cells transfected with the ezrin expression plasmid alone. Westerm immunoblotting of the cell lystaes was performed using the anti-ezrin (upper panel), anti-VSV-G epitope (middle panel), or anti-actin (lower panel) antibody. Panel B. The transfected cells were inoculated with the HIV-1 vector having the NDK Env protein. Relative values to transduction titer in the siRNA-GFP-transfected cells were indicated. This experiment was independently repeated three times. Error bars show standard deviations. Asterisks indicate statistical significance (P b 0.05).

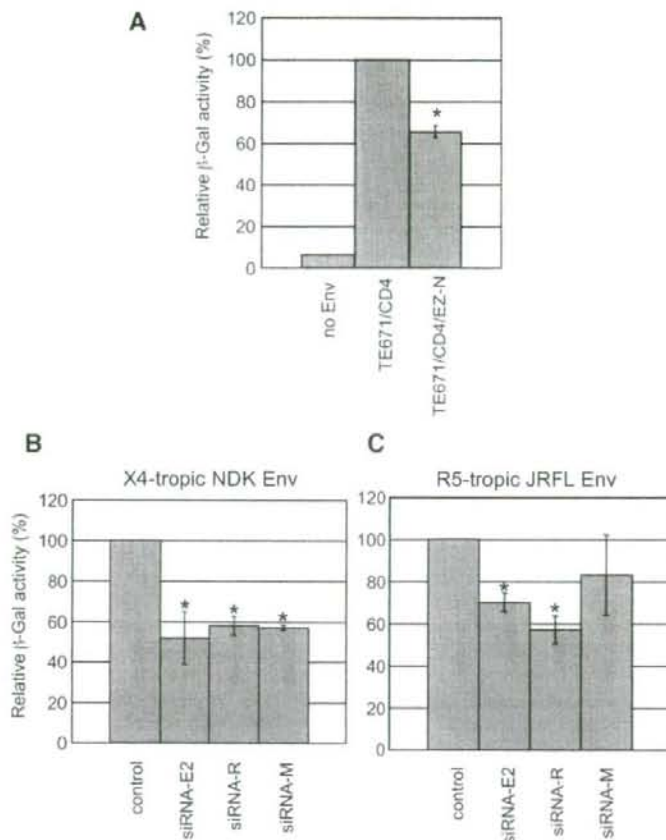

Fig. 6. Effect of ezrin dominant negative mutant and siRNAs against ERM family proteins on HIV-1-Env-mediated cell–cell fusion. 293T cells were transfected with the NDK (panels A and B) or JRFL (panel C) Env expression plasmid. TE671/CD4 or TE671/CD4/EZ-N cells transfected with the LTR-LacZ construct were added onto 293T cells transfected with the NDK Env expression plasmid (panel A). TE671/CD4/R5 cells were co-transfected with the each siRNA as indicated and the LTR-LacZ plasmid. The transfected TE671/CD4/R5 cells were added onto the transfected 293T cells. -Galactosidase activities of their cell lystaes were measured as described in Materials and methods. Relative values to -galactosidase activity of the mixed culture of the siRNA-GFP-transfected TE671/CD4/R5 cells and the Env-transfected 293T cells were indicated. This experiment was repeated three times, and error bars indicate standard deviations. Asterisks indicate statistical significance (P b 0.05).

which the authors showed that the moesin regulates stable microtubule formation and inhibits transduction of HIV-1 vectors having VSV-G protein in the rat cells (Naghavi et al., 2007). Our study deals with the similar topic but rather focused on the roles of the three ERM proteins in HIV-1-Env-mediated infections of human cells rather than VSV-Env-mediated infection of the rodent cells. Our study thus could reveal a hitherto unappreciated regulation mechanism, a pleiotropic regulation of HIV-1 infection by the ERM proteins.

Each of the siRNA against the ERM family proteins as well as the dominant negative mutant of ezrin inhibited transduction of X4-tropic HIV-1 vectors (Figs. 1B and 4B). These inhibitions were unlikely to be due to the reduced binding events of HIV-1 Env to the infection receptors, because the levels of cell surface expression of the CD4 and CXCR4 were similar in the ERM-suppression-positive and -negative cells (Figs. 1C and 4E). Similarly, the inhibitions were unlikely to be due to the overall reductions in the HIV-1 replication processes, because VSV-G-mediated HIV-1 transductions were not affected by the ERM suppression (Figs. 1B and 4A). Alternatively, our results strongly suggest that the ERM suppression induced specific inhibition of the X4-tropic HIV-1 infection at the entry step(s). Correlation between the X4-tropic vector transduction inhibition and cell–cell fusion inhibition (Figs. 4B and 6B) suggests that a key site of action for the siRNA-mediated inhibition is the membrane fusion. This in turn implies that the ERM proteins individually play positive roles in the membrane fusion mediated by interactions of X4-tropic Env and infection receptors.

Interestingly, transductions of the R5-tropic HIV-1 vectors were inhibited by the radixin siRNA alone, but were not by the ezrin and moesin siRNAs (Fig. 4C). The moesin siRNA rather increased the R5-tropic HIV-1 vector transductions. These results suggest that radixin of the ERM family is a key molecule for the efficient R5-tropic HIV-1 infection, whereas moesin rather

suppresses the HIV-1 R5 virus infection. Notably, such bimodal effects of the ERM proteins were not observed in the R5-tropic-Env-mediated cell–cell fusion (Fig. 6C). These results suggest that the ERM proteins including moesin function as positive regulators of R5-tropic-Env-mediated membrane fusion and that moesin additionally functions as a negative regulator of HIV-1 R5 virus replication at the early step(s) after the membrane fusion.

Our findings suggest that the ERM proteins regulate differently the R5- and X4-tropic HIV-1 infection. Underlying mechanisms by which the ERM proteins undergo the different regulation remain to be clarified. In this regard, CCR5 and CD4 co-localize on the plasma membrane before HIV-1 infection (Steffens and Hope, 2003, 2004), whereas CXCR4 and CD4 do not (Kozak et al., 2002). Such a difference in cell surface localization raises a possibility that regulation system for the CCR5 and CXCR4 fluidity on the plasma membrane is different. This in turn may lead to distinct regulation of infection receptor fluidity and cytoskeleton rearrangement by the ERM in CCR5 and CXCR4-mediated HIV-1 infection. However, we cannot exclude the possibility that the CCR5 over-expression in the target cells diminishes the ERM protein function for the R5-tropic HIV-1 infection (Jimenez-Baranda et al., 2007; Viard et al., 2002).

Recent study has reported that the moesin regulates stable microtubule formation and inhibits transduction of HIV-1 vectors having VSV-G protein in the rat cells (Naghavi et al., 2007). The findings suggest that moesin regulates cytoskeleton rearrangement to suppress HIV-1 replication somewhere after virus entry. Our data show that knockdown of moesin by siRNA resulted in enhancement of HIV-1 vector transduction only when the vector has the R5-tropic Env (Fig. 4C). The results suggest that in the case of R5-tropic virus infection, the moesin-mediated enhancement of infection is dominant in comparison with moesin-mediated suppression of HIV-1 replication, if any, after entry. The EZ-N protein suppressed the X4-tropic HIV-1 infection in TE671/CD4 and 293T/CD4 cells, but did not significantly in HeLa/CD4 cells (data not shown). Apparently the inconsistent results with HIV-1 vectors may imply differences of the moesin-mediated regulation system in these different cells, although further study is required for clarifying the issue.

The inhibitory effects of the ezrin dominant negative mutant and the ERM siRNAs on the HIV-1 vector transduction were not so high (about 50% reduction). As mentioned above, ERM proteins are highly homologous each other, and similarly functions, suggesting a possibility that other members of ERM family proteins complement functions of the proteins suppressed by the siRNA. Therefore, the target cells were simultaneously transfected with the siRNAs-E2, -R, and -M. However, the introduction of the three siRNAs resulted in severe cytotoxicity on the target cells as reported (Takeuchi et al., 1994), and it was difficult to analyze their effect on such cells. Transduction titers of the X4-tropic HIV-1 vectors on TE671/CD4 cells transfected with an siRNA against CXCR4, which actually reduced its expression level, was about 50% of those on the GFP siRNA-transfected cells (data not shown), like the ERM siRNAs. This result suggests that the ERM proteins function in X4-tropic HIV-1 entry as importantly as CXCR4 does.

In conclusion, we found that ezrin, radixin, and moesin proteins functions as pleiotropic regulators of HIV-1 infection in human cells. Our findings provide a basis to study HIV-1 entry in relation to the regulation of membrane protein fluidity and cytoskeleton rearrangement by the ERM proteins.

Materials and methods

Env protein expression plasmids

An X4-tropic HIV-1 NDK Env expression plasmid was kindly provided by Dr. U. Hazan (Dumonceaux et al., 1998). HIV-1 HXB2 (X4 tropic), JRFL (R5 tropic), NH1 (X4R5-tropic), and NH2 (R5 tropic) Env expression plasmids were kindly provided by Dr. Y. Yokomaku (Kusagawa et al., 2002; Yokomaku et al., 2004). These HIV-1 Env expression plasmids encodes HIV-1 tat and rev genes as well as the env sequence. A VSV-G expression plasmid (pHEF-VSV-G) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (Iwakuma et al., 1999).

Construction of C-terminally HA-tagged radixin expression plasmid

Total RNA samples were isolated from TE671 cells, and radixin cDNA was amplified by PCR using following primers; Rad-S (5 -GAGAAAGAAAATGCCGAAACC-3) and Rad-AS (5 -ATATATGCAAAATAACAGCTCTCA-3). The radixin PCR products were ligated into pTarget vector plasmid (Promega) by TA cloning. The predicted amino acid sequence of the radixin cDNA was completely identical to that of already reported human radixin. The radixin sequence was amplified by PCR using the Rad-S and Rad-HA (5 -TCATGCGTAATCCGGAACATCGTACGGGTATCCCATTGCTTCAAACTCATC-3) for C-terminal HA tagging. The antisense Rad-HA primer contains the HA tag sequence. The PCR product was ligated into pTarget vector and its nucleotide sequence was confirmed.

HIV-1 vector

A DNA construct (R8.91) that encodes HIV-1 proteins required for HIV-1 vector preparation except for Env protein was kindly provided by Dr. D. Trono (Naldini et al., 1996). A LacZ-containing HIV-1 vector genome expression plasmid (pTY-EFnLacZ) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (Chang et al., 1999).

Cells

Human TE671, 293T, HeLa cell lines were cultured at 37 C under 5% CO_2 in Dulbecco's modified Eagle's medium (Wako) supplemented with 8% fetal bovine serum (Biosource). CD4-expressing TE671 and HeLa cells (TE671/CD4 and HeLa/CD4) were constructed by transfection with a CD4-expression plasmid containing the neomycin resistant gene. CD4-expressing 293T cells (293T/CD4) were constructed by transfection with a CD4-expression plasmid containing the hygromycin resistant gene. TE671 cells expressing CD4 and CCR5 (TE671/CD4/R5) were