Ezrin, Radixin, and Moesin (ERM) Proteins Function as Pleiotropic Regulators of Human Immunodeficiency Virus Type 1 infection

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Running title: ERM proteins in HIV-1 infection

Keywords: HIV-1, ezrin, radixin, moesin

Abstract: 211 words. Figure: 6

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.

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 co-receptor (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 co-receptor binding domain. The binding of SU subunit to the co-receptor 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, Hildreth, and Schwartz, 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 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, Louvard, and Arpin, 2006; Tsukita, Yonemura, and Tsukita, 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, Yonemura, and Tsukita, 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, Curto, and McClatchey, 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 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 colocalize 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, Yonemura, and Tsukita, 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) 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, Ishimoto, and Amanuma, 2003). These functions of the ecotropic MLV Env proteins were not affected by the EZ-N 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. Figure 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 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 β-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 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; Steffens and Hope, 2004), whereas CXCR4 and CD4 do not (Kozak, Heard, and Kabat, 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-VSVG) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (Iwakuma, Cui, and Chang, 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 constructed as follows. The TE671/CD4 cells were inoculated with a CCR5 and puromycin resistant gene-encoding murine leukemia virus (MLV) vector constructed as reported (Kubo, Ishimoto, and Amanuma, 2003), and were selected with puromycin. Puromycin-resistant cell pool was used in this study. TE671 and 293T cells expressing CD4 and a C-terminally VSV-G-tagged dominant negative mutant of ezrin (EZ-N) was constructed by

inoculation of the TE671/CD4 and 293T/CD4 cells with an EZ-N-encoding MLV vector constructed as reported (Kubo, Ishimoto, and Amanuma, 2003), and designated as TE671/CD4/EZ-N and 293T/CD4/EZ-N. The VSV-G-tagged EZ-N plasmid was kindly provided from Dr. M. Arpin.

Transduction assay.

To obtain HIV-1 vector particles, 293T cells (5×10^5) were plated onto a 10-cm dish and cultured for 2 days. The 293T cells were transfected with the R8.91, pTY-EFnLacZ, and one of HIV-1 Env expression plasmids. The transfected 293T cells were washed to remove the transfection complex 24 hr after transfection, and continued to be cultured in fresh medium for additional 24 hr. Culture supernatants of the transfected cells were diluted to make their titer about 60 blue-cell-forming units per a microscopic field in ezrin dominant negative mutant-free or siRNA-free cells, and were inoculated into target cells. Target cells (2×10^5) were plated onto a 6-cm dish and were inoculated 24 hr after the plating. The inoculated cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Wako) 2 days after inoculation. Numbers of blue cells were counted to estimate transduction titers.

Western immunoblotting.

Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (BioRad), and were transferred onto a PVDF membrane (Millipore). The membrane was treated

with an anti-VSV-G (Sigma), -ezrin, -radixin, -moesin, or –actin antibody (Santa Cruz), and then with a horseradish peroxidase-conjugated protein G (BioRad). Protein G-bound polypeptides were visualized by ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

FACS.

To analyze cell surface expression of CXCR4 and CCR5, suspended cells were treated with a rat anti-CXCR4 or -CCR5 antibody (Tanaka et al., 2001). The cells were washed with PBS 3 times, and then treated with an FITC-conjugated anti-rat IgG antibody (Sigma). The cells were applied to a flow cytometer (Coulter). To analyze CD4 cell surface expression, cells were treated with an FITC-conjugated anti-CD4 antibody (Sigma).

Transfection of siRNA.

Sequences of sense strands of two siRNAs against ezrin were GAAUCCUUAGCGAUGAGAUCU (siRNA-E1) and CCUGAUUCUCGCGAUUAUUCU (siRNA-E2). Sequences of sense strands of siRNAs against radixin and moesin were CGACAAGUUAACACCUAAAU (siRNA-R) and CUCCCAGACGGAUCUGUUGC (siRNA-M). An siRNA against green fluorescence protein (GFP) was used as control, and sequence of the sense strand was CUGGAGUUGUCCCAAUUCUUG. These siRNAs were synthesized by RNAi Co. LTD. Cells were transfected with one of these siRNAs (200 pmol) by the TransIT TKO reagent (10 μl) (Mirus). To knockdown expression of all three ERM family proteins simultaneously, cells

were co-transfected with three siRNAs (total 600 pmol) by the transfection reagent (30 μl).

Semi-quantitative RT-PCR.

Total RNA was isolated from siRNA-transfected cells. First strand cDNA was synthesized from the total RNA with ramdom hexamer by a reverse transcriptase (TAKARA). Semi-quantitative PCR was performed using the first strand cDNA as template to detect ezrin, radixin, moesin, and GAPDH mRNAs. Nucleotide sequences of the PCR primers for ezrin mRNA were GCACAAACTTACCAG and TGGTCCTGGCCTGGCTGTTA, for radixin mRNA were GGCAACACAAAGCTTTTGCAG and ATATATGCAAAATAACAGCTCTCA, for moesin mRNA were TGAGGCTGTGGAGTGGCAGCA and CTAGAGGCTGGGTGCCCATTA, and for GAPDH mRNA were AGGTCGGAGTCAACGGATTTGGT and GTGGGCCATGAGGTCCACCAC. These primers were synthesized by Genenet Co. LTD.

Cell-fusion assay.

Receptor protein-expressing cells (2 X 10^5) were plasted onto a 6-cm dish and cultured for 2 days. The cells were co-transfected with the siRNA and a β-galactosidase expression plasmid, in which the β-galactosidase gene is under the control of an HIV-1 long terminal repeat (LTR-LacZ), by the TransIT TKO transfection reagent (Mirus). 293T cells (2 X 10^5) were plated onto a 6-cm dish and cultured for 2 days. The cells were transfected with a Tat expression plasmid as control or the HIV-1 Env expression plasmid by the Lipofectamine transfection reagent (Invitrogen). The

Env expression plmasids additionally encodes the tat gene. The transfected recipient cells (5 X 10^5) were added onto the HIV-1 Env-expressing 293T cells 24 hr after transfection. If these cells fuse, the Tat protein activates the β-galactosidase expression. β-Galactosidase activity of cell lysates was measured 24 hr after mixed culture by the high sensitive β-galactosidase activity kit (Stratagene).

Statistical analysis.

Differences between two groups of data were determined by the Student's t-test. Statistical significance was set at $p<0.05$ for all tests.

Acknowledgements

We thank Dr. U. Hazan for the NDK Env expression plasmid, Dr. Y. Yokomaku for the HXB2, JRFL, NH1, and NH2 Env expression plasmids, Dr. D. Trono for the R8.91 plasmid, Dr. L. Chang for the VSV-G expression plasmid and pTY-EFnLacZ, and Dr. M. Arpin for the expression plasmids of the wild type and dominant negative forms of ezrin. The VSV-G, TAT, and LacZ-containing HIV-1 vector genome expression plasmids were onbatined through AIDS Research and Reference Reagent Program, NIAID, NIH, USA. This study was supported by a Health Science Research Grant from the Ministry of Health, Labour, and Welfare of Japan and by the 21st century Centers of Excellence (COE) program.

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Figure legends

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.

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.

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.

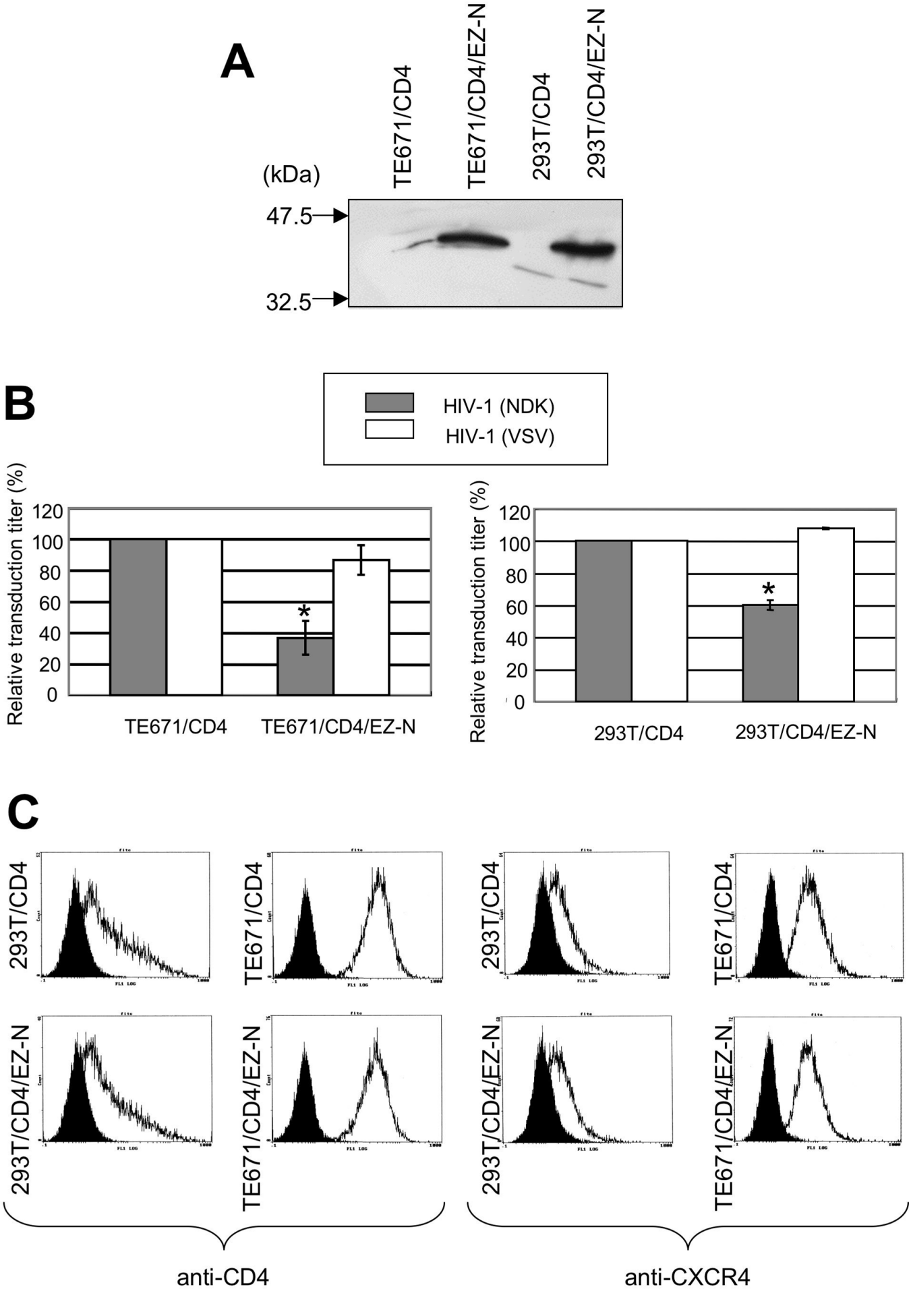
Fig. 4. **Effect of siRNAs against ERM family genes on HIV-1 vector transduction.** HIV-1 vector pseudotyped with VSV-G (panel A), HIV-1 X4-tropic Env (panel B), or HIV-1 R5-tropic Env (panel C) was inoculated into TE671/CD4/R5 cells transfected with siRNA-GFP, siRNA-E1, siRNA-R, or siRNA-M. HIV-1 vector 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 ($P<0.05$). 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. 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 ($P<0.05$).

Fig. 6. **Effect of ezrin dominant negative mutant and siRNAs against ERM family prteins 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. β-Galactosidase activities of their cell lystaes were measured as described in Materials and Methods. Relative values to β-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 ($P<0.05$).


A
TE671/CD4
TE671/CD4/EZ-N
293T/CD4
293T/CD4/EZ-N
(kDa)
47.5
32.5
B
HIV-1 (NDK)
HIV-1 (VSV)
Relative transduction titer (%)
120
100
80
60
40
20
0
*
TE671/CD4
TE671/CD4/EZ-N
293T/CD4
293T/CD4/EZ-N
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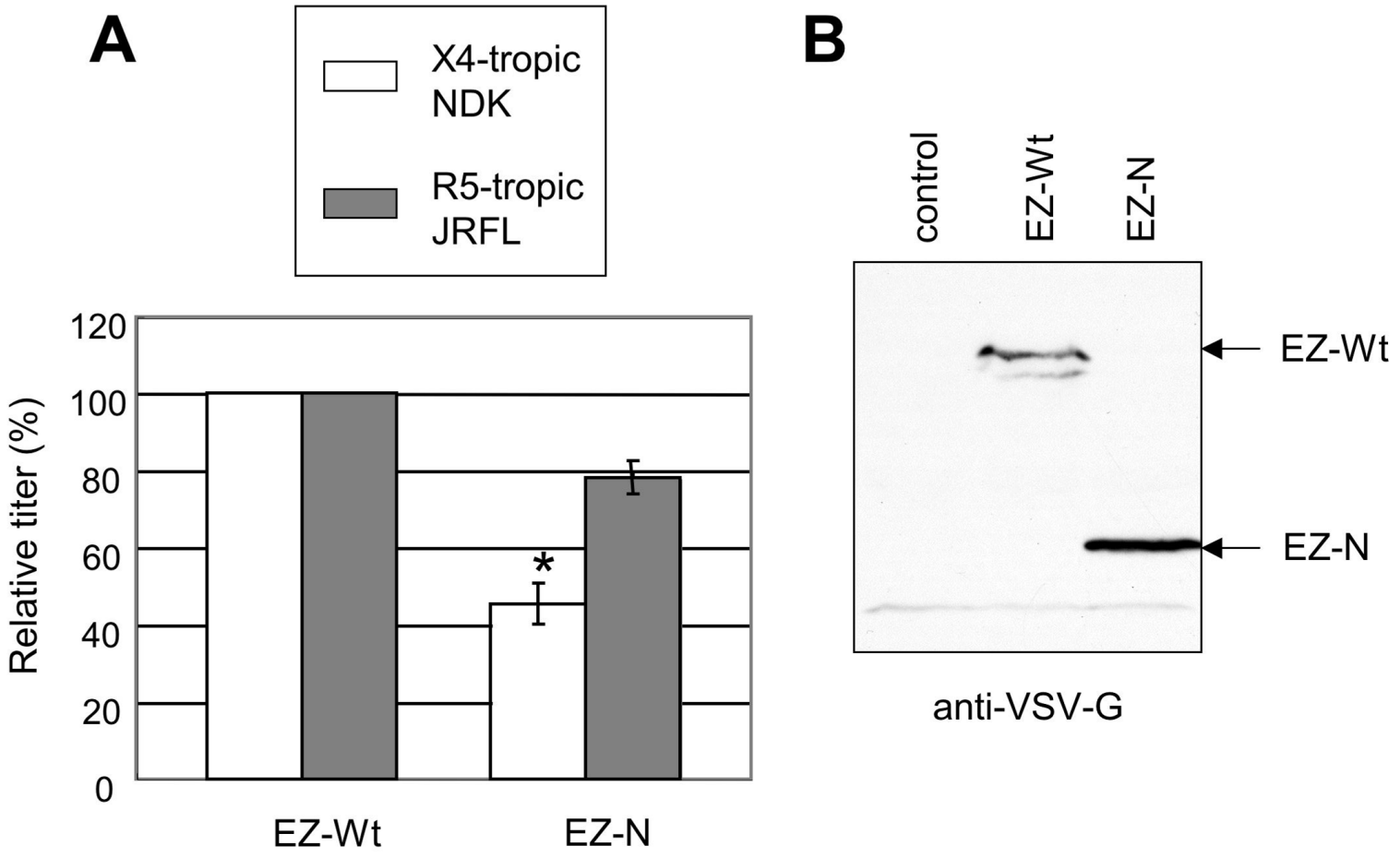
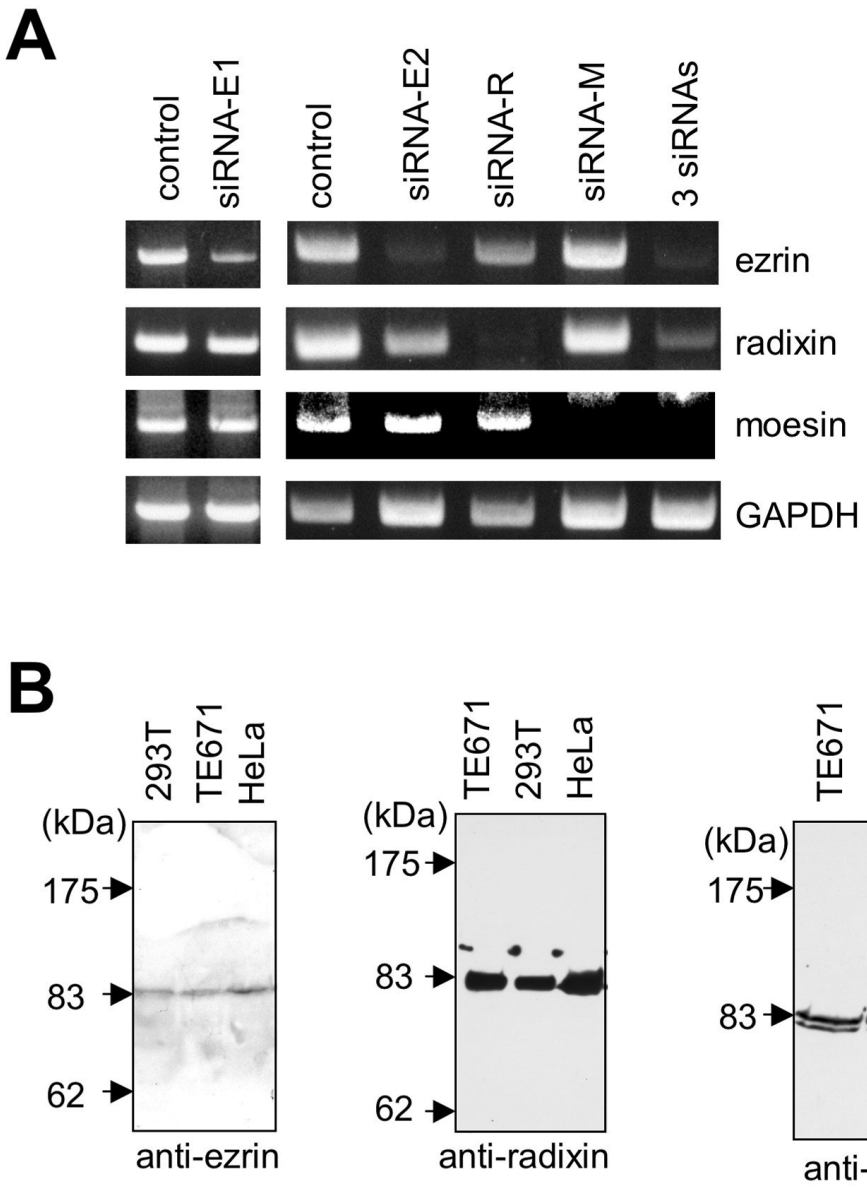
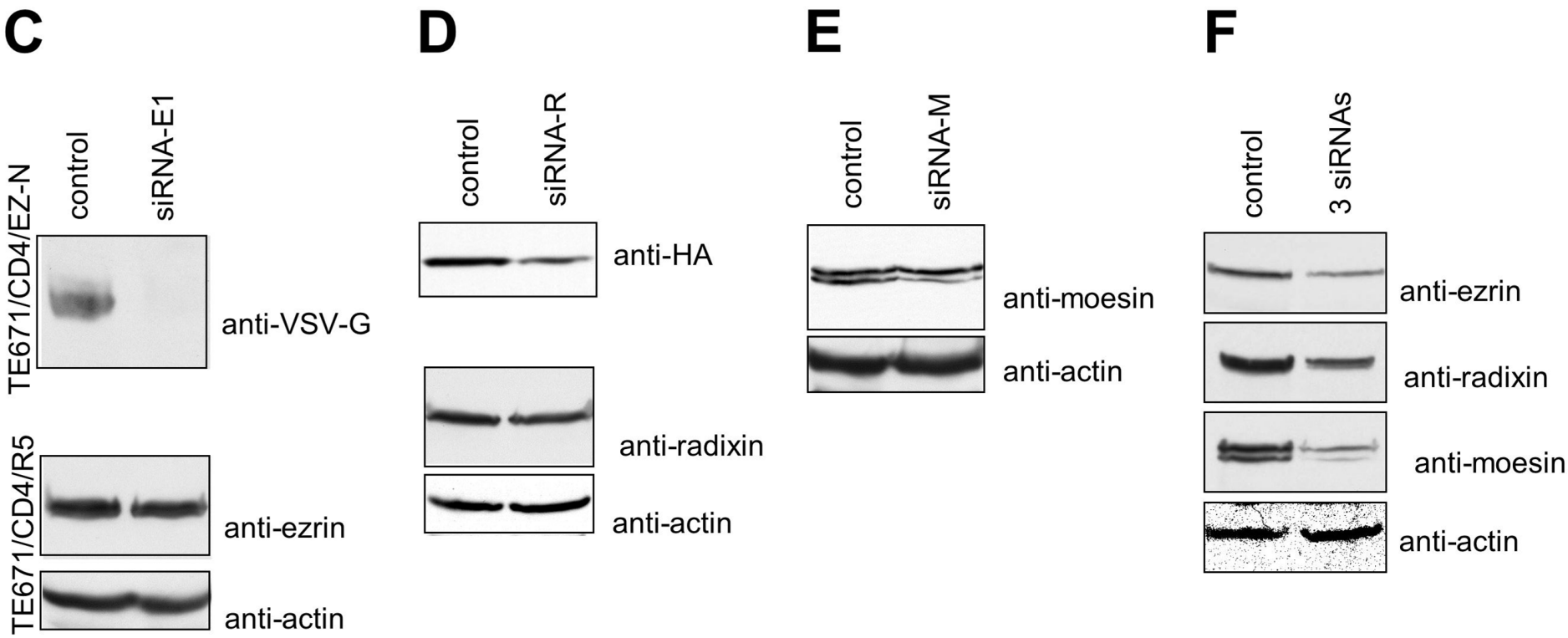
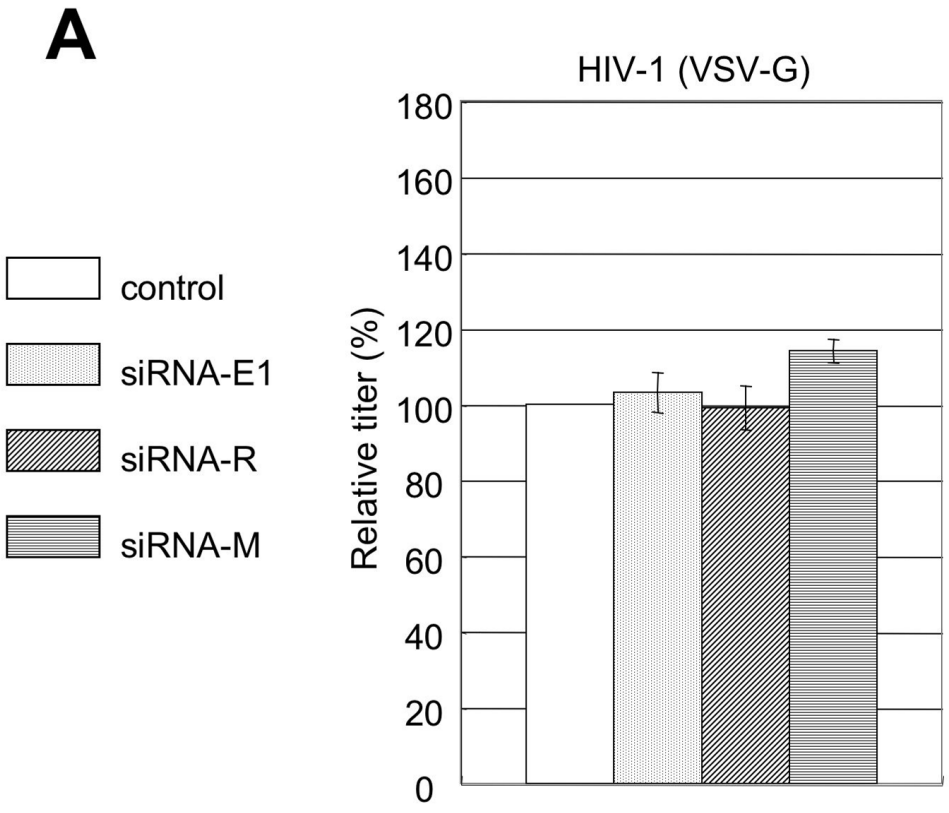
293T/CD4
TE671/CD4
293T/CD4/EZ-N
TE671/CD4/EZ-N
anti-CD4
anti-CXCR4

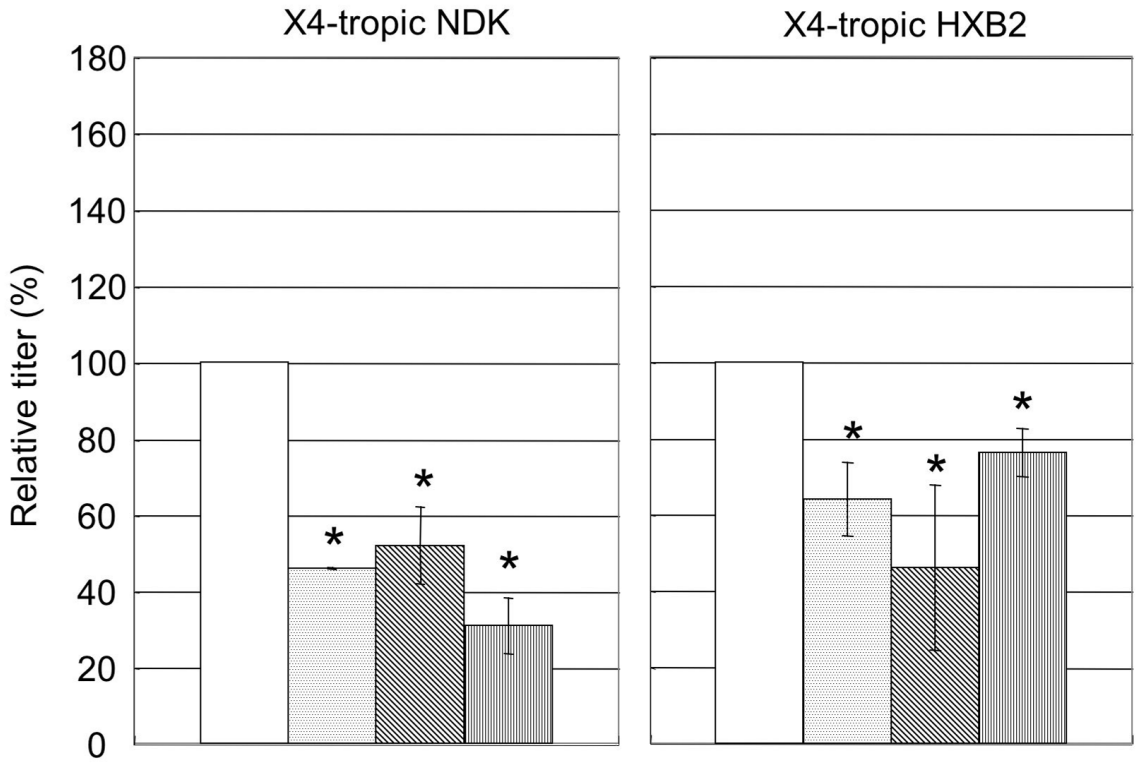

A
X4-tropic
NDK
R5-tropic
JRFL
Relative titer (%)
120
100
80
60
40
20
0
*
EZ-Wt
EZ-N
B
control
EZ-Wt
EZ-N
EZ-Wt
EZ-N
anti-VSV-G

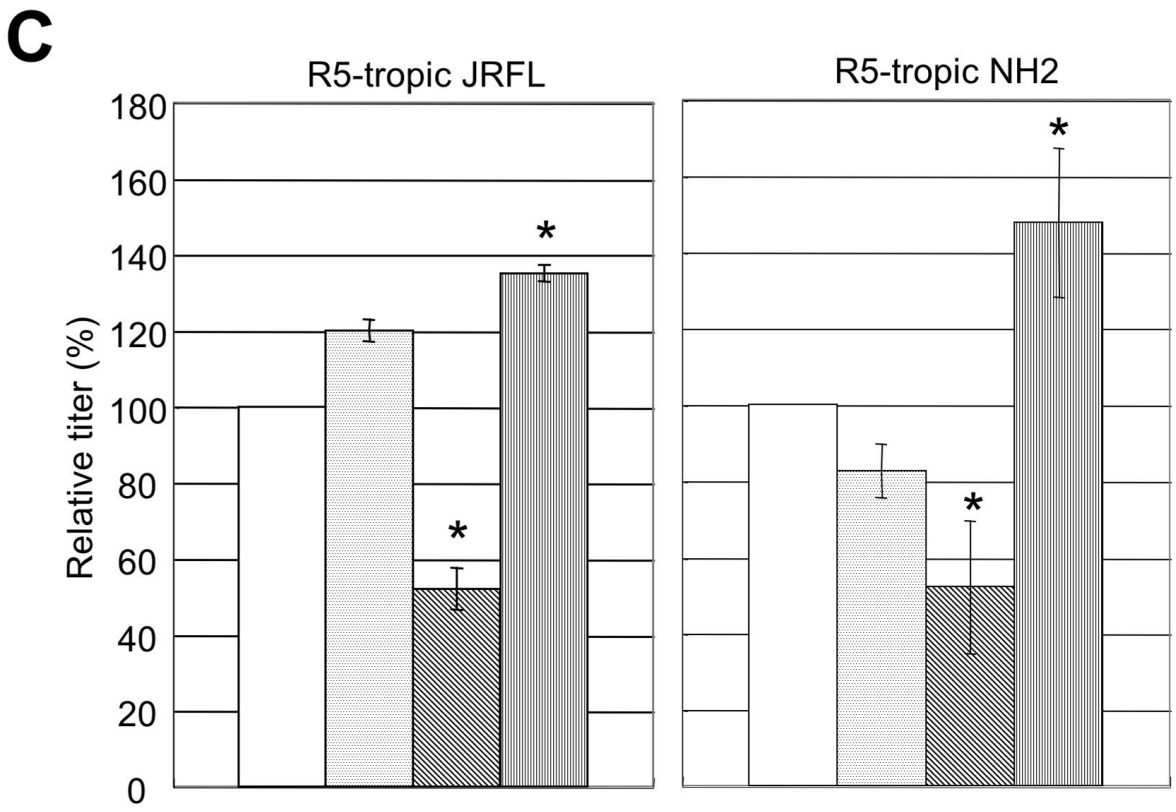

A
control
siRNA-E1
control
siRNA-E2
siRNA-R
siRNA-M
3 siRNAs
ezrin
radixin
moesin
GAPDH
B
293T
TE671
HeLa
(kDa)
175
83
62
anti-ezrin
TE671
293T
HeLa
(kDa)
175
83
62
anti-radixin
TE671
HeLa
293T
(kDa)
175
83
anti-moesin

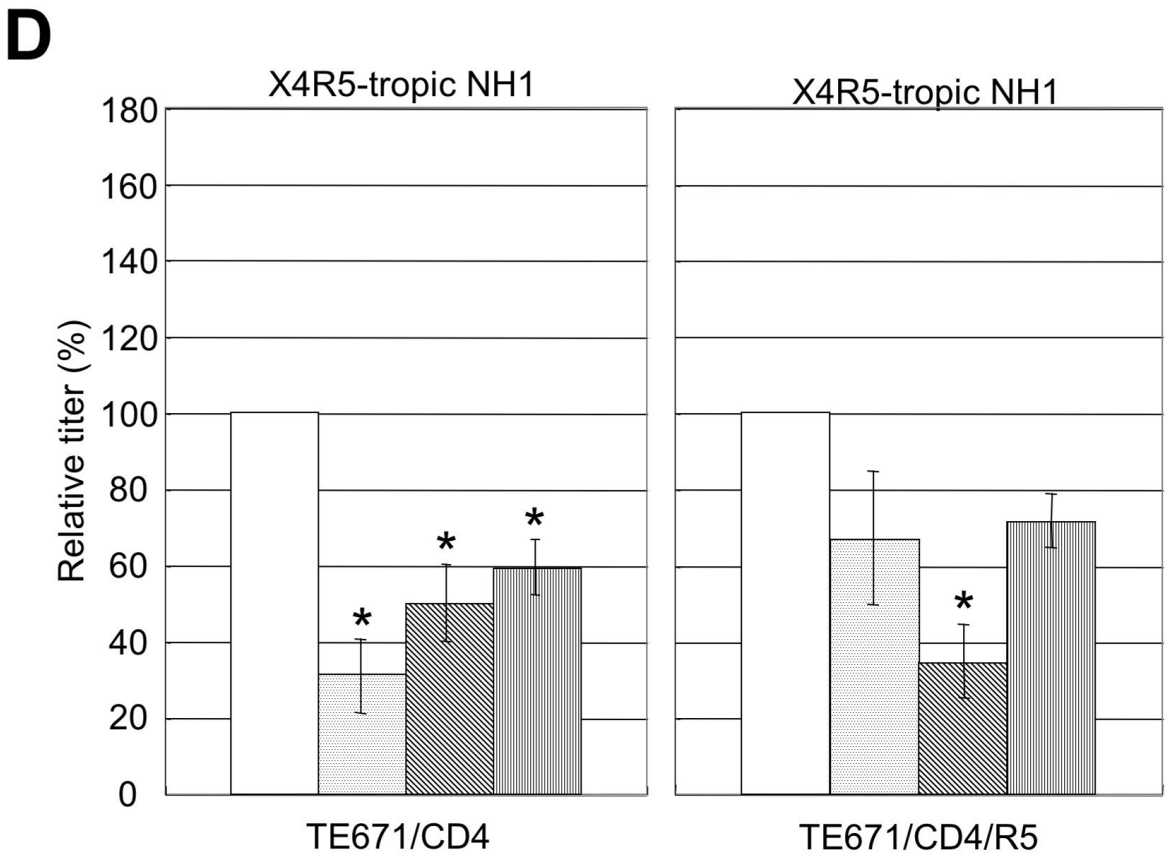

C
control
siRNA-E1
TE671/CD4/EZ-N
anti-VSV-G
TE671/CD4/R5
anti-ezrin
anti-actin
D
control
siRNA-R
anti-HA
anti-radixin
anti-actin
E
control
siRNA-M
anti-moesin
anti-actin
F
control
3 siRNAs
anti-ezrin
anti-radixin
anti-moesin
anti-actin


A
control
siRNA-E1
siRNA-R
siRNA-M
HIV-1 (VSV-G)
Relative titer (%)
180
160
140
120
100
80
60
40
20
0

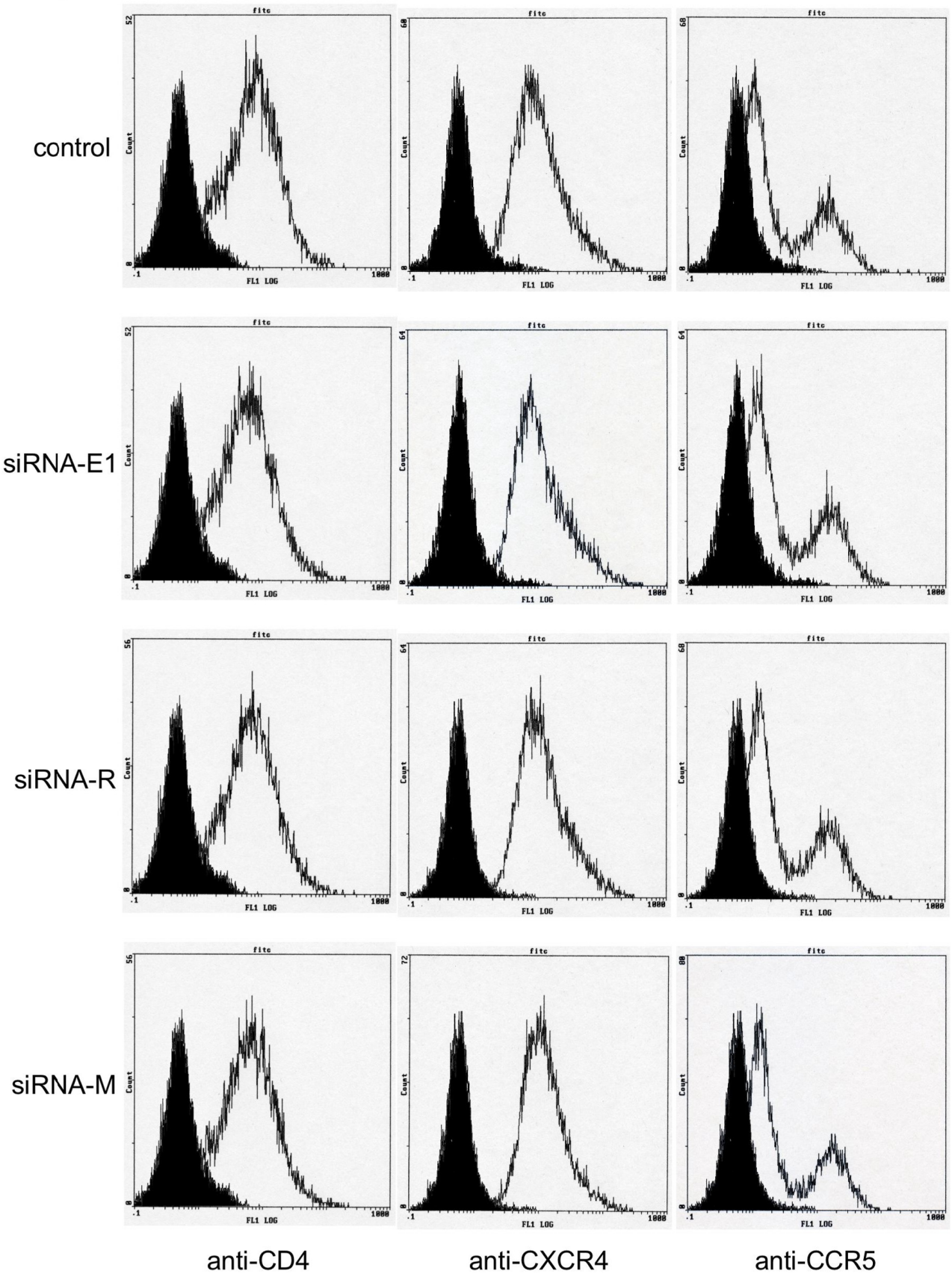
B


X4-tropic NDK
X4-tropic HXB2
Relative titer (%)
180
160
140
120
100
80
60
40
20
0
*
*
*
*
*
*


C
R5-tropic JRFL
R5-tropic NH2
Relative titer (%)
180
160
140
120
100
80
60
40
20
0
*
*
*
*


D
X4R5-tropic NH1
X4R5-tropic NH1
Relative titer (%)
180
160
140
120
100
80
60
40
20
0
*
*
*
*
TE671/CD4
TE671/CD4/R5

E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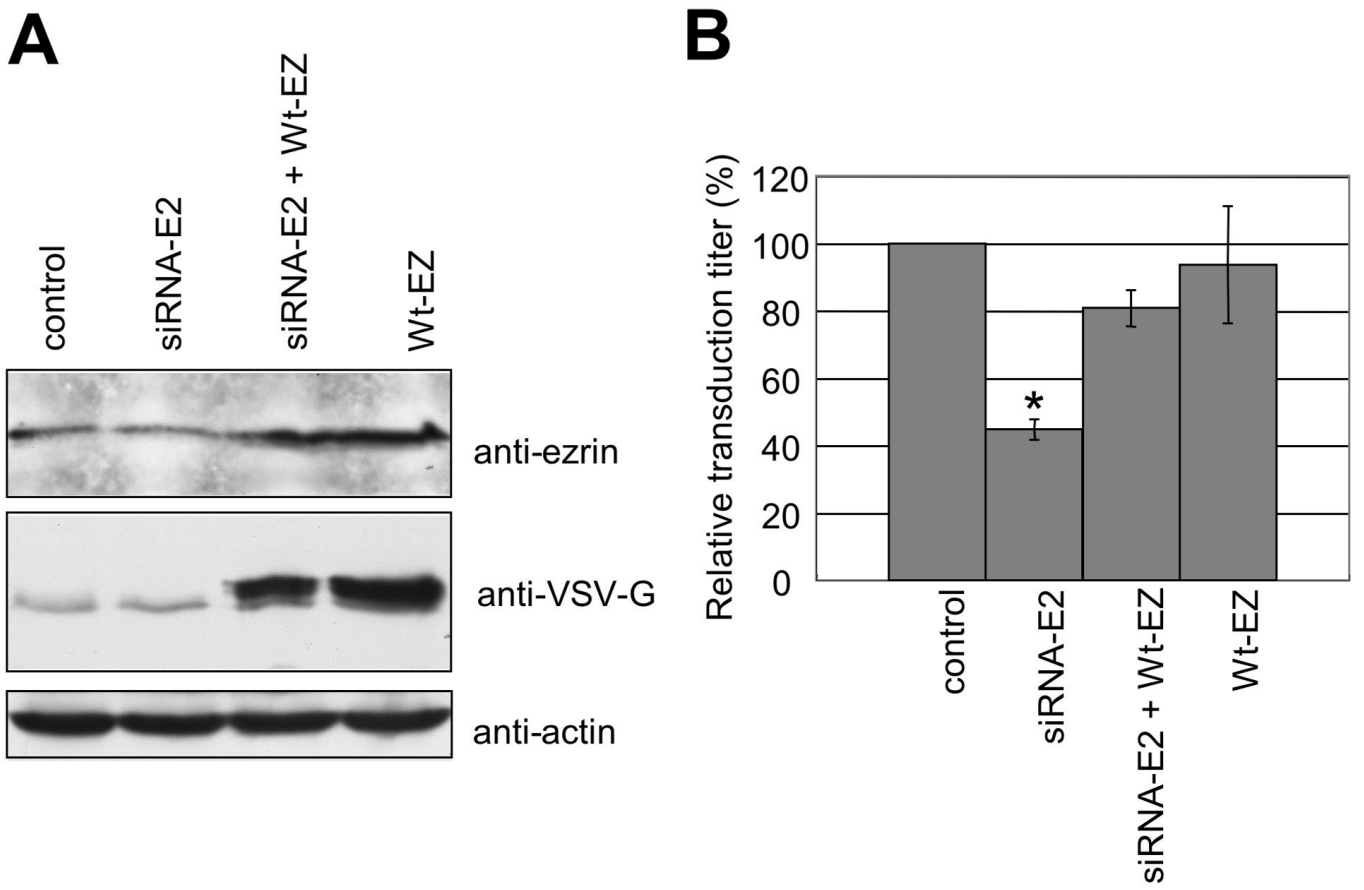
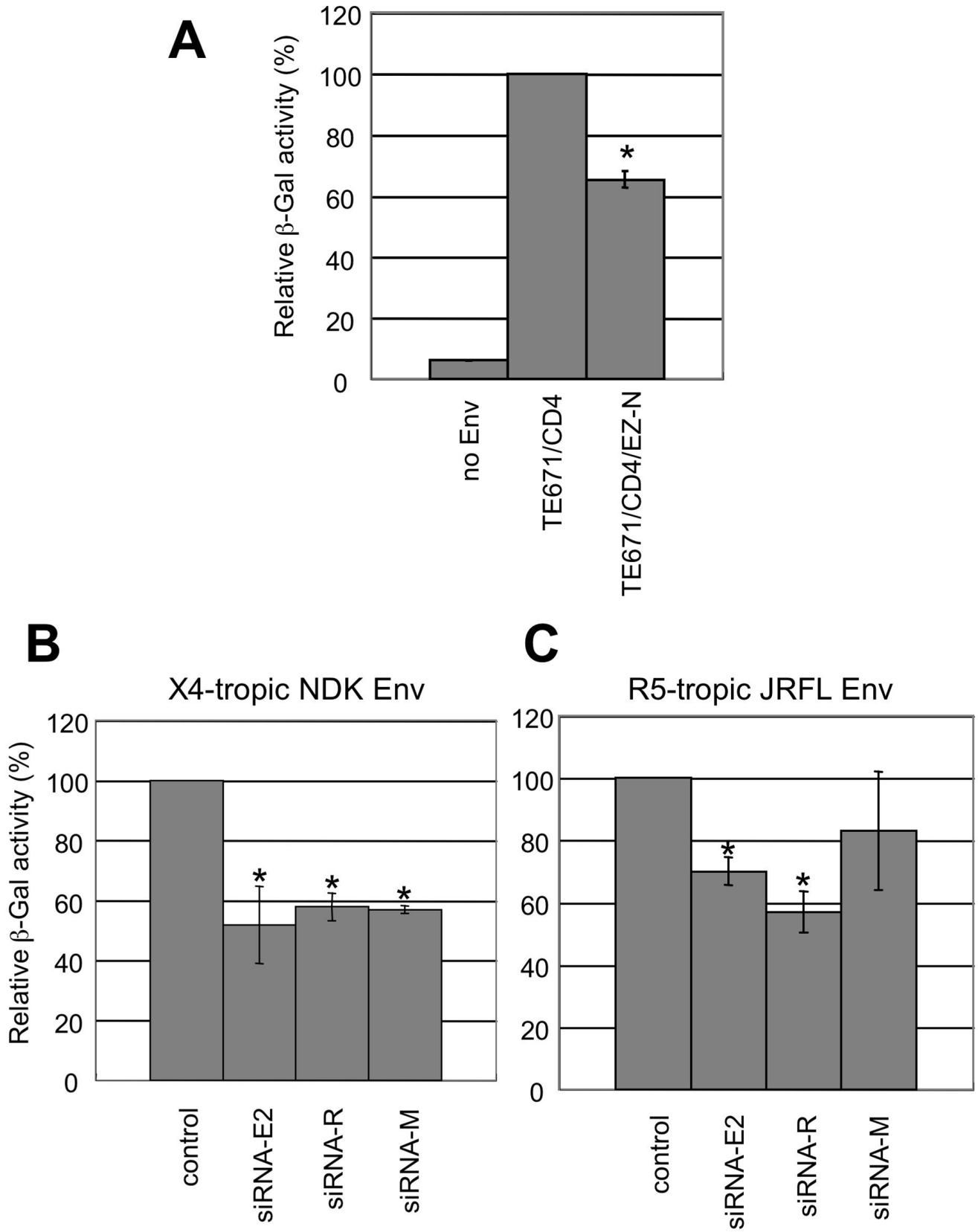
siRNA-E1
siRNA-R
siRNA-M
anti-CD4
anti-CXCR4
anti-CCR5
fitc
Count
FL1 LOG


A
control
siRNA-E2
siRNA-E2 + Wt-EZ
Wt-EZ
anti-ezrin
anti-VSV-G
anti-actin
B
Relative transduction titer (%)
120
100
80
60
40
20
0
*
control
siRNA-E2
siRNA-E2 + Wt-EZ
Wt-EZ


A
Relative β-Gal activity (%)
120
100
80
60
40
20
0
*
no Env
TE671/CD4
TE671/CD4/EZ-N
B
X4-tropic NDK Env
Relative β-Gal activity (%)
120
100
80
60
40
20
0
*
*
*
control
siRNA-E2
siRNA-R
siRNA-M
C
R5-tropic JRFL Env
120
100
80
60
40
20
0
*
*
control
siRNA-E2
siRNA-R
siRNA-M