Virology 532 (2019) 82–87

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Cytoplasmic R-peptide of murine leukemia virus envelope protein negatively regulates its interaction with the cell surface receptor

Yoshinao Kubo[a,*], Mai Izumida[b], Kei Togawa[a], Fengmin Zhang[c], Hideki Hayashi[d]

[a] Program for Nurturing Global Leaders in Tropical Medicine and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[b] Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan
[c] Department of Microbiology, Harbin Medical University, Harbin, China
[d] Medical University Research Administrator, Nagasaki University School of Medicine, Nagasaki, Japan

ARTICLE INFO

Keywords:
Murine leukemia virus
Receptor binding
Envelope protein
Transmembrane subunit
R-peptide

ABSTRACT

Cytoplasmic tails of envelope (Env) glycoproteins of many retroviruses inhibit their membrane fusion activity. The cytoplasmic 16-amino acid peptide of ecotropic murine leukemia virus (E-MLV) Env protein, called the R-peptide, also inhibits the membrane fusion activity of the Env protein. However, the molecular mechanism of the inhibition has not been elucidated yet. In this study, we found that R-peptide-containing Env protein of E-MLV binds to the cell surface receptor cationic amino acid transporter-1 (CAT-1) with weaker affinity than R-peptide-truncated Env protein. Consistent with this result, R-peptide-containing Env protein had less efficient inhibition of E-MLV vector infection than R-peptide-truncated Env protein. R-peptide truncation has been reported to induce conformational change in the surface subunit of E-MLV Env protein that interacts with the receptor. Taken together, our findings indicate that R-peptide truncation induces conformational change in the receptor-binding domain of the E-MLV Env protein and facilitates the Env–receptor interaction.

1. Introduction

The detailed mechanism of murine leukemia virus (MLV) replication has not been elucidated yet. MLV entry into host cells is mediated by its envelope (Env) glycoprotein. The Env protein is synthesized as a precursor, and then cleaved by a cellular protease into surface (SU) and transmembrane (TM) subunits. Furthermore, the C-terminal cytoplasmic 16-amino acid peptide of TM subunit, R-peptide, is cleaved by the viral protease after the virions are released (Green et al., 1981; Henderson et al., 1984; Shultz and Rein, 1985). SU binds to its cognate cell surface receptor, cationic amino acid transporter-1 (CAT-1) (Albritton et al., 1989; Wang et al., 1991; Kim et al., 1991), and TM induces fusion between virus and host membranes (Kubo et al., 2012).

R-peptide-truncated [R (−)] Env protein induces cell-cell fusion in susceptible cells, but R-peptide-containing [R (+)] Env protein does not (Ragheb and Anderson, 1994; Rein et al., 1994; Yang & Compans, 1996a, 1996b; Januszeski et al., 1997; Kim et al., 2000; Melikyan et al., 2000; Kubo et al., 2007; Taylor and Sanders, 2003). This finding shows that R-peptide inhibits membrane fusion mediated by the MLV Env protein. Since the R-peptide is cleaved after virions are released, MLV-producing cells express R (+) Env protein, and cell-cell fusion does not occur. Previously, we reported that Env mutants, whose R-peptides could not be cleaved, do not induce infection, suggesting that the fusogenicity activated by R-peptide cleavage is required for MLV infection (Kubo and Amanuma, 2003). Taken together, these findings indicate that MLV Env proteins contain R-peptide to maintain MLV progeny production through inhibition of fusion-mediated death of MLV-producing cells. After MLV particles are released, the R-peptide is cleaved to activate the membrane fusion activity required for viral entry.

The mechanism of R-peptide inhibition of membrane fusion has not been elucidated. Cannon's research group has studied cell surface expression of R (+) and R (−) Env proteins by flow cytometry using two different monoclonal antibodies, 273 and 83A25, that recognize N− and C-terminal regions of the SU subunit, respectively (Aguilar et al., 2003). Although similar levels of their cell surface expression were detected using the 83A25 antibody, the cell surface expression level of R (−) Env was higher than that of R (+) Env using the 273 antibody. Their finding suggests that R-peptide truncation induces conformational change in the N-terminal SU subunit. Garoff's research group has also shown that the R-peptide regulates the conformation of SU-TM

* Corresponding author. Program for Nurturing Global Leaders in Tropical Medicine and Emerging Communicable Diseases, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, 852-8523, Japan.
E-mail address: yoshinao@nagasaki-u.ac.jp (Y. Kubo).

https://doi.org/10.1016/j.virol.2019.04.005
Received 4 February 2019; Received in revised form 15 April 2019; Accepted 15 April 2019
Available online 23 April 2019
0042-6822/

complex using cryoelectron microscopy (Loving et al., 2012). However, how the conformational change affects membrane fusion activity has not been elucidated. Garoff's research group has also reported that the R-peptide controls disulfide exchange after the interaction between the E-MLV Env and mouse CAT-1 (Loving et al., 2008), and Melikyan's research group has shown that R (+) Env induces hemifusion but not fusion pore after binding of Env-expressing cells to target cells at 4 °C (Melikyan et al., 2000), because the R-peptide is linked to the TM subunit. However, these discoveries do not reveal the mechanism of membrane fusion activation by R-peptide truncation.

During analysis of cell fusion by the R (−) Env protein, we found that the R (−) Env protein of E-MLV can induce cell fusion, similar to that in uninfected cells, in E-MLV-producing cells. Although transduction titers of replication-defective E-MLV vectors in E-MLV-producing cells were significantly lower than those in uninfected cells, E-MLV vector-transduced cells were still detected. As mentioned above, E-MLV-producing cells express R (+) Env protein. Therefore, we hypothesized that R (−) Env protein binds to the cell surface receptor more efficiently than R (+) Env protein. In this study, we assessed this hypothesis.

2. Results

2.1. E-MLV R(−) Env protein induces cell-cell fusion in E-MLV-producing cells

To determine whether or not the R (−) Env of E-MLV induces cell fusion in E-MLV-producing cells, 293T cells were transfected with ecotropic R (+) or R (−) Env expression plasmids and mixed with uninfected or Moloney E-MLV-producing NIH3T3 cells. Surprisingly, fused cells were detected in E-MLV-producing cells (Supplementary Fig. 1A), although numbers of fused cells were moderately lower than those in uninfected cells.

To quantify cell fusion biochemically, 293T cells were transfected with the HIV-1 Tat expression plasmid together with pcDNA3.1 or R (−) Env expression plasmid; these cells were then mixed with uninfected or E-MLV-producing NIH3T3 cells that were transfected with the LacZ expression plasmid under the control of the HIV-1 long terminal repeat (LTR-LacZ) (Kubo et al., 2008; Kamiyama et al., 2009, 2011b). The Tat protein enhances transcription from HIV-1 LTR. If Tat-expressing cells fuse with the LTR-LacZ-containing cells, LacZ activity is elevated. LacZ activity in E-MLV-producing cells was similar to that in uninfected cells (Supplementary Fig. 1B). These results showed that R (−) Env induces cell fusion in E-MLV-producing cells.

2.2. E-MLV vector transduces E-MLV-producing cells

The above result indicated that the R (+) Env protein expressed in the E-MLV-producing cells did not inhibit cell fusion induced by the R (−) Env of E-MLV. This result prompted us to speculate that R (−) Env more efficiently binds to R (+) Env. This speculation suggested that E-MLV can infect to E-MLV-producing cells, although it is already known that E-MLV infection is strongly attenuated in E-MLV-producing cells, called receptor interference. To examine the speculation, TELCeB6 cells that stably express both MLV Gag-Pol and the LacZ-encoding MLV vector genome (Cosset et al., 1995) were transfected with the Wt E-MLV Env expression plasmid to construct LacZ-encoding E-MLV vector-producing cells. Uninfected and E-MLV-producing NIH3T3 cells were inoculated with serially diluted culture supernatants from the LacZ-encoding E-MLV vector-producing cells. The inoculated cells were stained with X-Gal, and blue cells were counted to estimate transduction titers. Transduction titers of culture supernatants from the E-MLV vector-producing cells were about 1×10^5 and 1×10^3 transduction units (TUs)/ml in uninfected and E-MLV-producing NIH3T3 cells, respectively (Supplementary Fig. 2A). Although transduction titers in the E-MLV-producing NIH3T3 cells were much lower than those in uninfected NIH3T3 cells, E-MLV vector-infected cells were detected in the E-MLV-producing NIH3T3 cells.

Cells expressing Env protein at a low level have been shown to exist in MLV-producing cells (Boi et al., 2017). The E-MLV vector might infect such cells. To investigate this speculation, E-MLV-producing NIH3T3 cells were inoculated with a puromycin resistance gene-encoding E-MLV vector, and the inoculated cells were selected using puromycin. Western blotting using anti-SU antibodies analyzed E-MLV Env expression levels in the puromycin-resistant cell pools and the original E-MLV-producing cells. SU levels in the puromycin-resistant cell pools were similar to those in the original E-MLV-producing cells (Supplementary Fig. 2B).

E-MLV-producing cells were inoculated with puromycin resistant gene-encoding E− or amphotropic (A)-MLV vector, and then were selected with puromycin. Viral titers of culture supernatants from these puromycin-resistant cell pools were also similar (Supplementary Fig. 2C). These results indicated that the E-MLV vector can infect the E-MLV-producing cells, even though the E-MLV Env protein was expressed.

2.3. R(+) Env less efficiently binds to the receptor-expressing cells than R (−) Env

As described above, E-MLV-producing cells express R (+) Env protein, and R (−) Env exists in MLV particles. It is unlikely that the cell surface receptor unoccupied by the Env protein still exist in the E-MLV-producing cells, because amount of Env protein is generally much higher than that of the receptor protein, and transduction titers of E-MLV vector in E-MLV-producing cells were indeed much lower than those in uninfected cells. Thus, we hypothesized that R (+) Env less efficiently binds to CAT-1 than R (−) Env. To assess this hypothesis, MLV vector particles containing R (+) Env were prepared. An E-MLV Env mutant containing an amino acid substitution of isoleucine for the leucine residue at the N-terminal side of the R-peptide cleavage site was used (L616I mutant). Our previous studies and that of another independent research group have showed that the L616I Env protein does not undergo R-peptide cleavage (Kubo and Amanuma, 2003; Loving et al., 2008). TELCeB6 cells (Cosset et al., 1995) were transfected with Wt or L616I E-MLV Env expression plasmid. In addition, the expression plasmid encoded a neomycin resistance gene. Thus, the transfected cells were selected with geneticin. Geneticin-resistant cell pools were used in the following experiments. The transduction titers of culture supernatants from the Wt and L616I Env-expressing TELCeB6 cells were approximately 1×10^5 and 2×10^2 TU/ml as we already reported (Kubo et al., 2007). Culture supernatants from the Wt Env- and L616I mutant-expressing TELCeB6 cells were centrifuged through 20% sucrose to collect MLV vector particles. Pellets were analyzed by Western blotting using anti-p30, anti-SU, anti-TM, and anti-R-peptide antibodies. Levels of p30 and SU for Wt Env-containing vector particles were similar to those for L616I Env-containing particles (Supplementary Figs. 3A and B). This result showed that the L616I mutant did not affect MLV particle production and incorporation of Env proteins into virions. The molecular size of the TM subunit in the Wt Env-containing particles was lower than that in the L616I Env-containing particles, indicating that R-peptide cleavage of the L616I Env protein was inhibited (Supplementary Fig. 3C). Analysis of the vector particles by Western blotting using anti-R-peptide antibodies showed a band only in the L616I Env-containing particles (Supplementary Fig. 3D). These results confirmed previous reports showing that R-peptide cleavage did not occur in the L616I mutant (Kubo and Amanuma, 2003; Loving et al., 2008).

The effect of the L616I mutation on cell surface expression was analyzed by flow cytometry using anti-SU antibody. Mean fluorescent intensities (MFIs) of 293T cells transfected with Wt, R (−), or L616I Env mutant expression plasmids were similar (Supplementary Fig. 3E). This result showed that cell surface expressions of Env proteins were

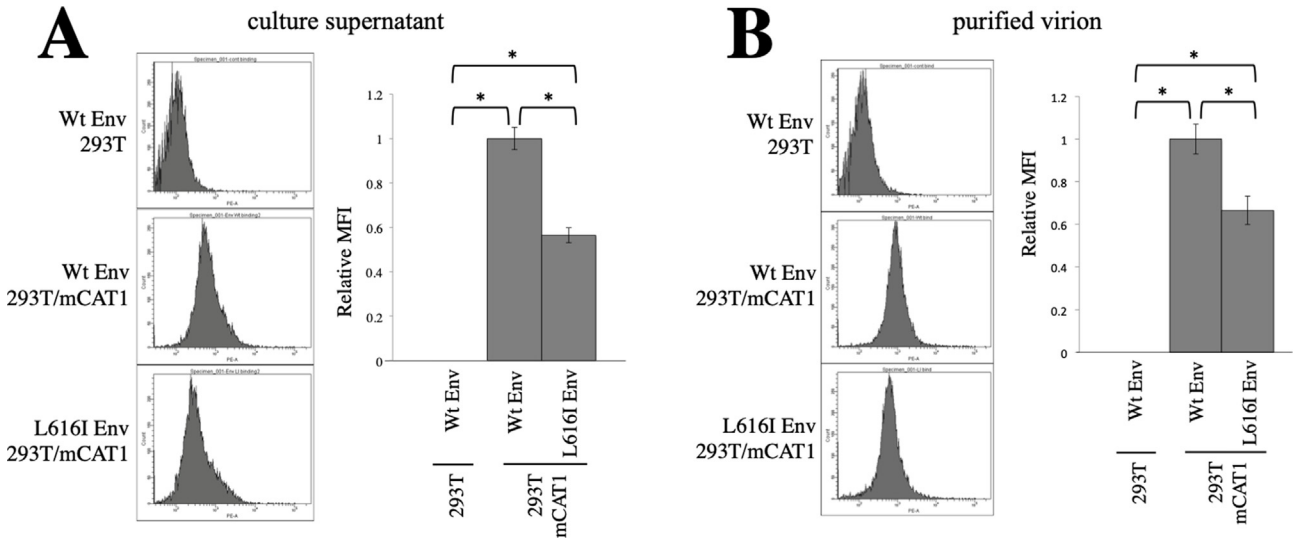

Fig. 1. L616I Env less efficiently bound to mouse CAT-1-expressing cells than wild type Env. 293T cells stably expressing mouse CAT-1 were treated with culture supernatants (A) or purified particles (B) from cells producing wild type Env- or L616I mutant-bearing MLV vector. The cells were incubated with goat anti-SU antiserum and then with PE-conjugated anti-goat IgG antibody. MFIs of the cells were measured by a flow cytometer. This experiment was independently repeated three times. MFIs of control 293T cells treated with Wt Env-containing MLV vector particles were subtracted from the MFIs presented. Relative values of subtracted MFIs in mCAT1-expressing 293T cells treated with Wt Env-containing MLV vector particles are shown. Error bars show SD.

not affected by the R-peptide truncation or L616I mutation.

To measure the interaction of Wt and L616I Env proteins with mouse CAT-1, 293T cells stably expressing mouse CAT-1 (293 T/mCAT1) were incubated with MLV vector particles containing Wt or L616I Env protein at 4 °C (Kamiyama et al., 2011a; Lavillette et al., 2001; Yoshii et al., 2008). As binding of E-MLV vector particle to NIH3T3 cells is weak due to a low CAT1 expression level, 293 T/mCAT1 cells were used in this experiment. The cells were treated with goat anti-SU antiserum and then with PE-conjugated anti-goat IgG antibody. Since The N-terminal domain of SU interacts with the cell surface receptor and the anti-SU antibody binds the C-terminal region of SU (Kubo et al., 2007), it may have no effect on interaction between the Env and receptor proteins. MFIs in cells incubated with the Wt R (−) Env-containing MLV vector particles were much higher than those with the L616I (R+) Env-containing particles (Fig. 1A), although cell surface expression of R (−), R (+), and L616I Env proteins were similar analyzed by flow cytometer using this anti-SU antibody (Supplementary Fig. 3E). MFIs of control 293T cells incubated with the Wt R (−) Env-containing MLV vector were much lower than those of CAT-1-expressing 293T cells.

Culture supernatants from the MLV vector-producing cells contain free SU subunits, and they may affect the binding of MLV particles to the cell surface receptor. To exclude this possibility, MLV particles carrying Wt or L616I Env protein were isolated by centrifugation through 20% sucrose cushion. The pellets were suspended with fresh medium and incubated with 293 T/mCAT1 cells. Similar to the above result, MFIs of 293 T/mCAT1 cells incubated with Wt Env-carrying MLV vector were higher than those with L616I Env-containing MLV vector (Fig. 1B). These results indicated that the L616I R (+) Env-containing MLV particles, in comparison with the Wt R (−) Env protein-containing particles, had less efficient binding to mouse CAT-1-expressing 293T cells.

If L616I R (+) Env protein indeed was less efficiently bound to CAT-1 than Wt Env, L616I R (+) Env would less efficiently inhibit infection by the Wt R (−) Env-containing vector than Wt R (−) Env. To investigate this speculation, a luciferase-encoding MLV vector containing the Wt R (−) Env protein was constructed by transient transfection of 293T cells with Wt Env, Gag-Pol, and luciferase-encoding MLV vector genome expression plasmids. Culture supernatants from the transfected cells were mixed with an equal volume of culture supernatant from TELCeB6 cells stably producing LacZ-encoding MLV vectors with Wt Env or the L616I mutant. Viral titers of culture supernatants from TELCeB6 cells stably expressing Wt Env protein and from 293T cells transiently transfected with LacZ-encoding E-MLV vector construction plasmids were approximately 1×10^5 and 2×10^4 TU/ml, respectively. Therefore, it was thought that amount of LacZ-encoding E-MLV vector particles was 5-fold higher than that of luciferase-encoding E-MLV vector particles in the mixed E-MLV vector solution. The mixed vector solution was inoculated to NIH3T3 cells (MOI of LacZ-encoding E-MLV vector = 1). When cells inoculated with the mixture of LacZ-encoding and luciferase-encoding Wt E-MLV vectors were stained with X-Gal, almost all cells were blue. When luciferase activities in the inoculated cells were measured, the L616I mutant less efficiently decreased luciferase activities of inoculated cells in comparison with the Wt Env (Fig. 2A), although p30 amounts in their virion preparations were similar (Supplementary Fig. 3A). When culture supernatants from the luciferase-encoding E-MLV vector were mixed with those from amphotropic MLV vector-producing TELCeB6 cells (MOI = 1), luciferase activity was slightly reduced.

To assess dose-dependent effect of Wt or L616I Env-containing MLV vector, culture supernatants from the TELCeB6 cells producing Wt or L616I Env-containing vector were diluted with fresh medium, and were mixed with luciferase-encoding E-MLV vector (5:1). The mixed vector solutions were inoculated to NIH3T3 cells, and luciferase activities were measured. Even when the culture supernatants were diluted with medium 1/4-fold, Wt R (−) Env-containing MLV particles, but not L616I R (+) Env-bearing particles, significantly inhibited luciferase-encoding E-MLV vector infection (Fig. 2B). These results supported the above conclusion that R (+) Env less efficiently bound to the cell surface receptor in comparison with R (−) Env.

3. Discussion

The inhibitory role of C-terminal tails of Env proteins in membrane fusion is conserved in many retroviruses, but the molecular mechanism is not yet known. In this study, we found that the C-terminal R-peptide tail of the MLV Env protein negatively regulates the interaction between the MLV SU and cell surface receptor proteins. Since the R-peptide is linked to the TM subunit and the TM subunit induces membrane fusion after cell surface receptor recognition, impacts of the

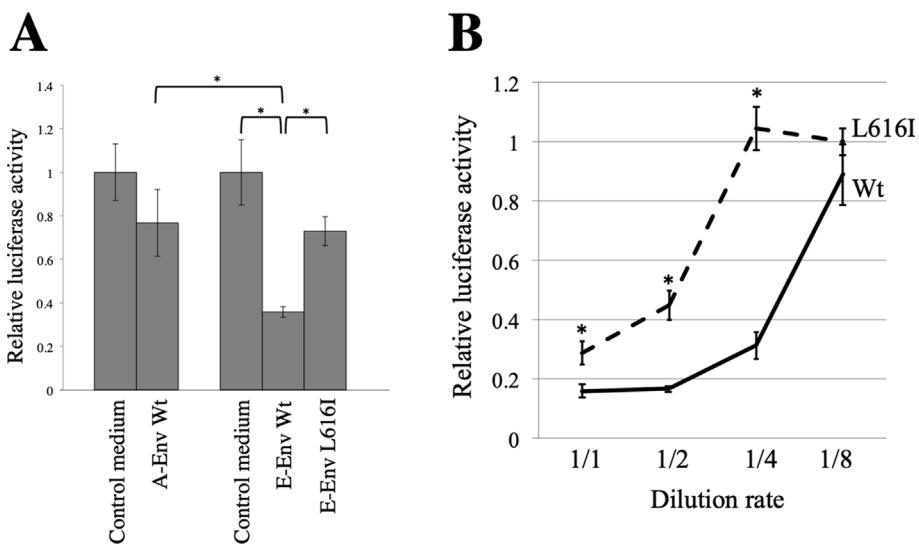

Fig. 2. L616I Env-carrying MLV less efficiently competes with E-MLV vector infection. Luciferase-encoding E-MLV vector was mixed with equal volume of culture supernatant from no Env (control medium), Wt amphotropic Env (A-Env Wt), Wt ecotropic Env (E-Env Wt), or L616I Env-containing E-MLV vector-producing TELCeB6 cells (panel A). Culture supernatant from Wt ecotropic or L616I Env-containing vector-producing cells was diluted with fresh medium as indicated, and mixed with luciferase-encoding E-MLV vector (5:1) (panel B). The mixed supernatants were inoculated onto NIH3T3 cells, and luciferase activities were measured. This experiment was independently repeated three times at different days. Relative values to luciferase activity in the control mixture-inoculated cells are indicated. Error bars show SD. Asterisks show significant differences between the indicated groups.

R-peptide on a virological event after receptor recognition have been studied (Loving et al., 2008; Melikyan et al., 2000). However, the R-peptide affects receptor recognition itself. R-peptide truncation in MLV TM has been shown to influence the conformation of the SU subunit (Aguilar et al., 2003; Loving et al., 2012). Taken together, the conformational change of the SU subunit by R-peptide truncation in the TM subunit may enhance its interaction with the cell surface receptor. As mentioned above in the introduction, R-peptide cleavage has been suggested to be required for MLV infection by activation of membrane fusion. This study found that R-peptide cleavage is required for MLV infection by enhancing the affinity between the Env and cell surface receptor proteins. Similar to the MLV Env protein, the cytoplasmic domains of Env proteins of other retroviruses regulate conformation of their cell surface ectodomain and receptor binding (Spies and Compans, 1994; Spies et al., 1994; Chen et al., 2015; Vzorov et al., 2015, 2016; Vzorov and Compans, 2016; Samal et al., 2018; Edwards et al., 2002; Saha et al., 2005; Huang et al., 2008; Dimonte et al., 2011). Thus, the receptor-binding pocket of the MLV Env protein may be exposed after R-peptide cleavage. Consistent with this speculation, recognition of the MLV Env N-terminus containing the receptor-binding pocket by the monoclonal antibody 273 is altered by R-peptide truncation, although recognition by the antibody 83A25 of the C-terminus is intact (Aguilar et al., 2003).

Cell fusion was induced by the R (−) Env protein of E-MLV in uninfected and E-MLV-producing cells at a similar extent. However, E-MLV-producing cells were much less susceptible to E-MLV vector infection than uninfected cells. Interaction areas between Env-expressing and target cells are much larger than those between virus and target cells, and the number of Env-receptor complexes formed in cell fusions is much higher than that in infections. Even if membrane fusion occurs at one point in the large interacting area, cell fusion is completed. Therefore, cell fusion was not inhibited in the E-MLV-producing cells.

R (−) Env-bearing MLV vector can infect to E-MLV-producing cells, although transduction titers were much decreased. R (+) Env is released from a part of the cell surface receptor molecules due to its low affinity, and R (−) Env protein may bind to the Env-free receptor in E-MLV-producing cells. Alternatively, R (−) Env protein may detach R (+) Env from the receptor and bind to the receptor. To understand the mechanism, further study is needed.

4. Materials and methods

4.1. Cells

Long-term cultures of human 293T and mouse NIH3T3 cells were maintained in our laboratory. Dr. Cossett kindly provided TELCeB6 cells that express the LacZ-encoding MLV vector genome and the MLV Gag-Pol protein (Cosset et al., 1995). These cells were cultured in Dulbecco's modified Eagle's medium (Wako) with 8% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich). To construct E-MLV-producing NIH3T3 cells, 293T cells were transfected with an infectious Moloney E-MLV expression plasmid (Bacheler and Fan, 1981) (1 μg) using Fugene transfection reagent (7 μl) (Roche) in a 6-cm dish, and the culture supernatant of the transfected cells was inoculated onto NIH3T3 cells in the presence of polybrene (4 μg/ml). The inoculated cells were maintained for more than one month. To construct mouse CAT-1-expressing 293T cells, 293T cells were transfected with mouse CAT1-encoding MLV vector genome, MLV Gag-Pol, and VSV-G expression plasmids (0.5 μg each). Culture supernatant of the transfected cells was inoculated onto 293T cells. Since the MLV vector additionally encodes the puromycin resistance gene, the inoculated cells were treated with puromycin. Puromycin-resistant cell pools were used in this study. Cells producing MLV vectors carrying the Wt Env or the L616I mutant were constructed as follows. TELCeB6 cells (Cosset et al., 1995) were transfected with the Env expression plasmid (1 μg). Since the expression plasmid additionally encodes the neomycin resistance gene, the transfected cells were selected with geneticin (Invitrogen). Geneticin-resistant cell pools were used in this study.

4.2. Plasmids

Plasmids expressing the R (+) and R (−) Env protein of Moloney E-MLV were previously constructed in our laboratory (Kubo and Amanuma, 2003). Mouse CAT-1-containing plasmids were obtained from Dr. J. M. Cunningham (Albritton et al., 1989). Mouse CAT-1-encoding MLV vector genome expression plasmids were previously constructed in our laboratory (Kubo et al., 2003). The MLV Gag-pol expression plasmid was purchased from TaKaRa. A luciferase-encoding MLV vector genome expression plasmid was constructed in this study.

4.3. Cell fusion assay

293T cells were transfected with R (+) or R (−) Env expression plasmids (1 μg). The transfected cells (1×10^6) were mixed with mouse NIH3T3 cells (1×10^6) in a 6-cm dish 24 h after the transfection. The cells were stained with 1% methylene blue 24 h after mixing and observed under a microscope (Olympus).

To quantify cell fusion biochemically, 293T cells were transfected with the HIV-1 Tat expression plasmid (0.5 μg) together with Wt or R (−) Env expression plasmids (0.5 μg). NIH3T3 cells were transfected

with the LacZ expression plasmid under the control of HIV-1 LTR (1 μg) (Kubo et al., 2008; Kamiyama et al., 2009, 2011b). These transfected cells (1×10^6 each) were mixed in a 6-cm dish 24 h after the transfection and further cultured for 24 h. LacZ activity of the cells was measured with the High Sensitivity β-galactosidase assay kit (Stratagene). Absorbance values at 570 nm were measured.

4.4. MLV particle binding assay

Mouse CAT-1-expressing 293T cells (1×10^7) were suspended with culture supernatants (4 ml) from confluent E-MLV vector-producing TELCeB6 cells and incubated at 4 °C for 2 h (Kamiyama et al., 2011a; Lavillette et al., 2001; Yoshii et al., 2008). The cells were washed with PBS, treated with goat anti-SU antiserum for 2 h, and then with PE-conjugated anti-goat IgG antibody. MFIs of the cells were measured using a flow cytometer. Values obtained after subtracting the MFIs of control cells are shown.

4.5. MLV vector infection

293T cells were transfected with luciferase-encoding MLV vector genome, Gag-Pol, and Wt E-MLV Env expression plasmids (0.5 μg each). The culture supernatant of transfected cells was replaced with fresh medium 24 h after the transfection, and cells were cultured for an additional 24 h. The culture supernatant from the transfected cells was mixed with an equal volume of culture supernatant from the Wt amphotropic Env-, Wt ecotropic Env-, or L616I mutant-expressing TELCeB6 cells. To achieve inoculation, culture supernatants of NIH3T3 cells (1×10^5) were completely replaced by the mixed supernatants (2 ml) in 12-well plates, and polybrene (Sigma-Aldrich) was added (4 μg/ml). The inoculated cells were stained with X-Gal 2 days after the inoculation, and numbers of blue cells were counted in seven randomly selected microscopic fields (1 mm^2). The luciferase activity of the inoculated cells was measured a luciferase activity assay kit (Promega).

4.6. Western blotting

Virion fractions were collected as follows. Culture supernatants (3 ml) were centrifuged at $280 \times g$ for 10 min to remove cells and cell debris and were further centrifuged at $1600 \times g$ for 4 h through 20% sucrose. The resulting pellets were suspended with 30 μl RIPA buffer and used as virion fractions. Cells (1×10^7) were lysed with 100 μl RIPA buffer. Cell lysates or virion fractions (15 μl) were mixed with 2x sample buffer (15 μl) and subjected to SDS-PAGE (Bio-Rad). The proteins were transferred onto PVDF membranes (Millipore). The membranes were treated with goat anti-SU (81S262; ViroMed), anti-p30 (81S263; ViroMed), anti-TM, or anti-R-peptide antiserum and then treated with HRP-conjugated anti-goat IgG antibody (Bio-Rad). The anti-TM and anti-R-peptide antisera were kindly provided by Dr. A. Rein (Shultz and Rein, 1985) and Dr. K. B. Andersen (Olsen and Andersen, 1999), respectively. These Western blotting experiments were performed on separate membranes. The antibody-bound proteins were visualized using ECL reagent (Bio-Rad).

Acknowledgements

We thank Dr. A. Rein for the anti-MLV TM antibody, Dr. K. B. Andersen for the anti-R-peptide antibody, Dr. E. Nishida for laboratory space and support, and Ms. A. Kondo for assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.04.005.

Conflicts of interest

We have no conflict of interest to disclose.

Grants

This study was supported by a grant-in-aid from the Japan Society for the Promotion of Sciences (15K08499), the Research Program on HIV/AIDS from the Japan Agency for Medical Research and Development (AMED) (JP17fk0410204), and the Asahi Kasei Medical Co., Ltd.

References

Aguilar, H.C., Anderson, W.F., Cannon, P.M., 2003. Cytoplasmic tail of Moloney murine leukemia virus envelope protein influences the conformation of the extracellular domain: implications for mechanism of action of the R peptide. J. Virol. 77, 1281–1291.

Albritton, L.M., Tseng, L., Scaden, D., Cunningham, J.M., 1989. A putative murine ecotropic receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57, 659–666.

Bacheler, L., Fan, H., 1981. Isolation of recombinant DNA clones carrying complete integrated proviruses of Moloney murine leukemia virus. J. Virol. 37, 181–190.

Boi, S., Dis, E.V., Hansen, E.J., Rosenke, K., Peterson, K.E., Ferrell, M.E., Evans, L.H., 2017. Latent murine leukemia virus infection characterized by the release of non-infectious virions. Virology 506, 19–27.

Chen, J., Kovacs, J.M., Peng, H., Rits-Volloch, S., Lu, J., Park, D., Zablowsky, E., Seaman, M.S., Chen, B., 2015. HIV-1 envelope. Effect of the cytoplasmic domain on antigenic characteristics of HIV-1 envelope glycoprotein. Science 349, 191–195.

Cosset, F.-L., Takeuchi, Y., Battini, J.L., Reiss, R.A., Collins, M.K., 1995. High-titer packaging cells producing recombinant retroviruses resistant to human serum. J. Virol. 69, 7430–7436.

Dimonte, S., Mercurio, F., Svicher, V., D'Arrigo, R., Perno, C.F., Ceccherini-Silberstein, F., 2011. Selected amino acid mutations in HIV-1 B subtype gp41 are associated with specific gp120v3 signatures in the regulation of co-receptor usage. Retrovirology 8, 33.

Edwards, T.G., Wyss, S., Reeves, J.D., Zolla-Pazner, S., Hoxie, J.A., Doms, R.W., Baribaud, F., 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. J. Virol. 76, 2683–2691.

Green, N., Shinnick, T.M., Witte, O., Ponticelli, A., Sutcliffe, J.G., Lerner, R.A., 1981. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. Proc. Natl. Acad. Sci. U.S.A. 78 6023-2027.

Henderson, L.E., Sowder, R., Copeland, T.D., Smythers, G., Oroszlan, S., 1984. Quantitative separation of murine leukemia virus proteins by reverse-phase high pressure liquid chromatography reveals newly described gag and env cleavage products. J. Virol. 52, 492–500.

Huang, W., Toma, J., Fransen, S., Stawiski, E., Reeves, J.D., Whitcomb, J.M., Parkin, N., Petropoulos, C.J., 2008. Coreceptor tropism can be influenced by amino acid substitutions in the gp41 transmembrane subunit of human immunodeficiency virus type 1 envelope protein. J. Virol. 82, 5584–5593.

Januszeski, M.M., Connan, P.M., Chen, D., Rozrnberg, Y., Anderson, W.F., 1997. Functional analysis of the cytoplasmic tail of Moloney murine leukemia virus envelope protein. J. Virol. 71, 3613–3619.

Kamiyama, H., Kakoki, K., Yoshii, H., Iwao, M., Igawa, T., Sakai, H., Hayashi, H., Matsuyama, T., Yamamoto, N., Kubo, Y., 2011a. Infection of XC cells by MLVs and Ebola virus is endosome-dependent but acidification-independent. PLoS One 6, e26180.

Kamiyama, H., Kubo, Y., Sato, H., Yamamoto, N., Fukuda, T., Ishibashi, F., Iwao, M., 2011b. Synthesis, structure-activity relationships, and mechanism of action of anti-HIV-1 lamellarin α 20-sulfate analogues. Bioorg. Med. Chem. 19, 7541–7550.

Kamiyama, H., Yoshii, H., Tanaka, Y., Sato, H., Yamamoto, N., Kubo, Y., 2009. Raft localization of CXCR4 is primarily required for X4-tropic human immunodeficiency virus type 1 infection. Virology 386, 23–31.

Kim, J.W., Closs, E.I., Albritton, L.M., Cunningham, J.M., 1991. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature 352, 725–728.

Kim, F.J., Seiliez, I., Denesvre, C., Lavillette, D., Cosset, F.L., Sitbon, M., 2000. Definition of an amino-terminal domain of the human T-cell leukemia virus type 1 envelope surface unit that extends the fusogenic range of an ecotropic murine leukemia virus. J. Biol. Chem. 275, 23417–23420.

Kubo, Y., Amanuma, H., 2003. Mutational analysis of the R peptide cleavage site of Moloney murine leukemia virus ebvelope protein. J. Gen. Virol. 84, 2253–2257.

Kubo, Y., Hayashi, H., Matsuyama, T., Sato, H., Yamamoto, N., 2012. Retrovirus entry by endocytosis and cathepsin proteases. Adv Virol 2012, 640894.

Kubo, Y., Ishimoto, A., Amanuma, H., 2003. N-linked glycosylation is required for XC cell-specific syncytium formation by the R peptide-containing envelope protein of ecotropic murine leukemia viruses. J. Virol. 77, 7510–7516.

Kubo, Y., Tominaga, C., Yoshii, H., Kamiyama, H., Mitani, C., Ananuma, H., Yamamoto, N., 2007. Characterization of R peptide of murine leukemia virus envelope glycoproteins in syncytium formation and entry. Arch. Virol. 152, 2169–2182.

Kubo, Y., Yoshii, H., Kamiyama, H., Tominaga, C., Tanaka, Y., Sato, H., Yamamoto, N., 2008. Ezrin, Radixin, and Moesin (ERM) proteins function as pleiotropic regulators of human immunodeficiency virus type 1 infection. Virology 375, 130–140.
Lavillette, D., Boson, B., Russell, S.J., Cosset, F.L., 2001. Activation of membrane fusion by murine leukemia viruses is controlled in cis or in trans by interactions between the receptor-binding domain and a conserved disulfide loop of the carboxy terminus of the surface glycoproteins. J. Virol. 75, 3685–3695.
Loving, R., Kronqvist, M., Sjoberg, M., Garoff, H., 2008. R-peptide cleavage potentiates fusion-controlling isomerization of the intersubunit disulfide in Moloney murine leukemia virus Env. J. Virol. 82, 2594–2597.
Loving, R., Wu, S.R., Sjoberg, M., Lindqvist, B., Garoff, H., 2012. Maturation cleavage of the murine leukemia virus Env precursor separates the transmembrane subunits to prime it for receptor triggering. Proc. Natl. Acad. Sci. U.S.A. 109, 7735–7740.
Melikyan, G.B., Markosyan, R.M., Brener, S.A., Rozenberg, Y., Cohen, F.S., 2000. Role of the cytoplasmic tail of ecotropic Moloney murine leukemia virus Env protein in fusion pore formation. J. Virol. 74, 447–455.
Olsen, K.E., Andersen, K.B., 1999. Palmitoylation of the intracytoplasmic R peptide of the transmembrane envelope protein in Moloney murine leukemia virus. J. Virol. 73, 8975–8981.
Ragheb, J.A., Anderson, W.F., 1994. pH-independent murine leukemia virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry. J. Virol. 68, 3220–3231.
Rein, A., Mirro, J., Haynes, J.G., Ernst, S.M., Nagashima, K., 1994. Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p12E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. J. Virol. 68, 1773–1781.
Saha, K., Yan, H., Nelson, J.A., Zerhouni-Layachi, B., 2005. Infection of human and non-human cells by a highly fusogenic primary CD4-independent HIV-1 isolate with a truncated envelope cytoplasmic tail. Virology 337, 30–44.
Samal, S., Das, S., Boliar, S., Qureshi, H., Shrivastava, T., Kumar, N., Goswami, S., Bansal, M., Chakrabarti, B.K., 2018. Cell surface ectodomain integrity of a subset of functional HIV-1 envelopes is dependent on a conserved hydrophilic domain containing region in their C-terminal tail. Retrovirology 15, 50.
Shultz, A., Rein, A., 1985. Maturation of murine leukemia virus env proteins in the absence of other viral proteins. Vriology 145, 335–339.
Spies, C.P., Compans, R.W., 1994. Effects of cytoplasmic domain length on cell surface expression and syncytium-forming capacity of the simian immunodeficiency virus envelope glycoprotein. Virology 203, 8–19.
Spies, C.P., Ritter, D.G., Mulligan, M.J., Compans, R.W., 1994. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein alters the conformation of the external domain. J. Virol. 68, 585–591.
Taylor, G.M., Sanders, D.A., 2003. Structure criteria for regulation of membrane fusion and virion incorporation by the murine leukemia virus TM cytoplasmic domain. Virology 312, 295–305.
Vzorov, A.N., Compans, R.W., 2016. Cytoplasmic domain effects on exposure of co-receptor-binding sites of HIV-1 Env. Arch. Virol. 161, 3011–3018.
Vzorov, A.N., Wang, L., Chen, J., Wang, B.Z., Compans, R.W., 2016. Effects of modification of the HIV-1 Env cytoplasmic tail on immunogenicity of VLP vaccines. Virology 489, 141–150.
Vzorov, A.N., Yang, C., Compans, R.W., 2015. An amphipathic sequence in the cytoplasmic tail of HIV-1 Env alters cell tropism and modulates viral receptor specificity. Acta Virol. 59, 209–220.
Wang, H., Kavanaugh, M.P., North, R.A., Kabat, D., 1991. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. Nature 352, 729–731.
Yang, C., Compans, R.W., 1996a. Analysis of the murine leukemia virus R peptide: Delineation of the molecular determinants which are important for its fusion inhibition activity. J. Virol. 71, 8490–8496.
Yang, C., Compans, R.W., 1996b. Analysis of the cell fusion activities of chimeric simian immunodeficiency virus-murine leukemia virus envelope proteins: inhibitory effects of the R peptide. J. Virol. 70, 248–254.
Yoshii, H., Kamiyama, H., Amanuma, H., Oishi, K., Yamamoto, N., Kubo, Y., 2008. Mechanisms underlying glycosylation-mediated loss of ecotropic receptor function in murine MDTF cells and implications for receptor evolution. J. Gen. Virol. 89, 297–305.