several possible explanations can be offered. First, those authors examined the localization of EGFP-G3BP within 48 h postinfection, and we observed it at later times (Fig. 4). Second, they used only EGFP-tagged G3BP instead of endogenous G3BP1. Third, they used a Jc1FLAG2 (p7-nsGluc2A) clone, and an HCV-JFH1 clone could markedly induce the recruitment of the core protein to LDs compared to that of Jc1. Also, Jangra et al. failed to observe the recruitment of DDX6 to LDs at 2 days after infection with HJ3-5 virus (16). Accordingly, we also observed that most of the DDX6 still formed intact P bodies at earlier times (12 h or 24 h postinfection). Importantly, we observed the recruitment of DDX6 to LDs 48 h later (Fig. 4). Furthermore, those authors did not show the ringlike structure formation of the HJ3-5 core protein around LDs, unlike the JFH1 core protein that we used in this study. The interaction of the HCV core protein with DDX6 may explain the recruitment of P-body components to LDs. However, we do not yet know whether the P-body function(s) can be performed on LDs. At least, HCV infection did not affect the translation of several host mRNAs with 5′ caps and 3′ poly(A) tails despite the disruption of P-body formation at 72 h postinfection (Fig. 6), suggesting that HCV does not affect P-body function and that HCV recruits functional P bodies to LDs.

We need to address the potential role of stress granule components, such as PABP1, in HCV replication/translation, since the HCV genome does not harbor the 3′ poly(A) tail. Intriguingly, we have found that the accumulation of HCV RNA was significantly suppressed in PABP1 knockdown RSc cells (Fig. 7F). In this regard, Tingting et al. demonstrated previously that G3BP1 and PABP1 as well as DDX1 were identified as the HCV 3′-UTR RNA-binding proteins by proteomic analysis and that G3BP1 was required for HCV RNA replication (35). Yi et al. also reported that G3BP1 was associated with HCV NS5B and that G3BP1 was required for HCV RNA replication (42). We observed a moderate effect of siG3BP1 on HCV RNA replication (Fig. 7F). In contrast, the accumulation of HCV RNA was significantly suppressed in ATX2 and Lsm1 knockdown cells as well as in PABP1 knockdown cells (Fig. 7F), suggesting that ATX2, Lsm1, and PABP1 are required for HCV replication.

Taking these results together, this study has demonstrated for the first time that HCV hijacks P-body and stress granule components around LDs. This hijacking may regulate HCV RNA replication and translation. Indeed, we have found that the accumulation of genome-length HCV-O (genotype 1b) (14) RNA was markedly suppressed in DDX6 knockdown O cells (data not shown). More importantly, these P-body and stress granule components may be involved in the maintenance of the HCV RNA genome without 5′ cap and 3′ poly(A) tail structures in the cytoplasm for long periods, since the hijacking of P-body and stress granule components by HCV occurred at later times.

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Glyceraldehyde 3-phosphate dehydrogenase negatively regulates human immunodeficiency virus type 1 infection

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Abstract

Background: Host proteins are incorporated inside human immunodeficiency virus type 1 (HIV-1) virions during assembly and can either positively or negatively regulate HIV-1 infection. Although the identification efficiency of host proteins is improved by mass spectrometry, how those host proteins affect HIV-1 replication has not yet been fully clarified.

Results: In this study, we show that virion-associated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) does not allosterically inactivate HIV-1 reverse transcriptase (RT) but decreases the efficiency of reverse transcription reactions by decreasing the packaging efficiency of lysyl-tRNA synthetase (LysRS) and $tRNA^{Lys3}$ into HIV-1 virions. Two-dimensional (2D) gel electrophoresis demonstrated that some isozymes of GAPDH with different isoelectric points were expressed in HIV-1-producing CEM/LAV-1 cells, and a proportion of GAPDH was selectively incorporated into the virions. Suppression of GAPDH expression by RNA interference in CEM/LAV-1 cells resulted in decreased GAPDH packaging inside the virions, and the GAPDH-packaging-defective virus maintained at least control levels of viral production but increased the infectivity. Quantitative analysis of reverse transcription products indicated that the levels of early cDNA products of the GAPDH-packaging-defective virus were higher than those of the control virus owing to the higher packaging efficiencies of LysRS and $tRNA^{Lys3}$ into the virions rather than the GAPDH-dependent negative allosteric modulation for RT. Furthermore, immunoprecipitation assay using an anti-GAPDH antibody showed that GAPDH directly interacted with $Pr55^{gag}$ and $p160^{gag\text{-}pol}$ and the overexpression of LysRS in HIV-1-producing cells resulted in a decrease in the efficiency of GAPDH packaging in HIV particles. In contrast, the viruses produced from cells expressing a high level of GAPDH showed decreased infectivity in TZM-bl cells and reverse transcription efficiency in TZM-bl cells and peripheral blood mononuclear cells (PBMCs).

Conclusions: These findings indicate that GAPDH negatively regulates HIV-1 infection and provide insights into a novel function of GAPDH in the HIV-1 life cycle and a new host defense mechanism against HIV-1 infection.

Keywords: Glyceraldehyde 3-phosphate dehydrogenase, Lysyl-tRNA synthetase, $tRNA^{Lys3}$, Human immunodeficiency virus type 1

Background

Because the HIV-1 genome only encodes a limited number of viral proteins, HIV-1 must take advantage of multiple functions of host proteins in order to successfully replicate. Several studies of purified HIV-1 virions have shown that in addition to proteins encoded by the virus, host proteins are found in the virions [1]. Some of these proteins may be taken into the virions simply because of their proximity to the viral assembly and budding sites, while other host proteins, such as cyclophilin A and LysRS, are included in HIV-1 particles as a result of their interaction with *gag* or *gag-pol* proteins during assembly [2-7]. These host proteins play an important role in facilitating the process of *gag* protein folding and $tRNA^{Lys3}$ packaging. Therefore, one way to elucidate the viral replication capacity gained by the packaging of host proteins is to directly analyze the host proteins inside the virions. A purified $HIV\text{-}1_{LAV\text{-}1}$ preparation was analyzed

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by 2D gel electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Proteome analysis demonstrates that GAPDH is inside the virions.

GAPDH is a prototype "moonlighting" protein that is involved in glycolysis, the carbon reduction cycle, the exportation of nuclear RNA, DNA repair, the bundling of microtubules, and apoptosis [8-14]. Furthermore, GAPDH also regulates viral replication. Interestingly, GAPDH phosphorylates the hepatitis B virus core protein [15] and binds with the *cis*-acting RNAs of several viruses, such as the 5′ UTR of the hepatitis A virus [16], the 3′ UTR sequences of the human parainfluenza virus [17], and the hepatitis C virus [18]. These findings indicated that GAPDH might also play a role in regulating HIV-1 replication.

In this study, we show that GAPDH is incorporated into the virions and the suppression of GAPDH

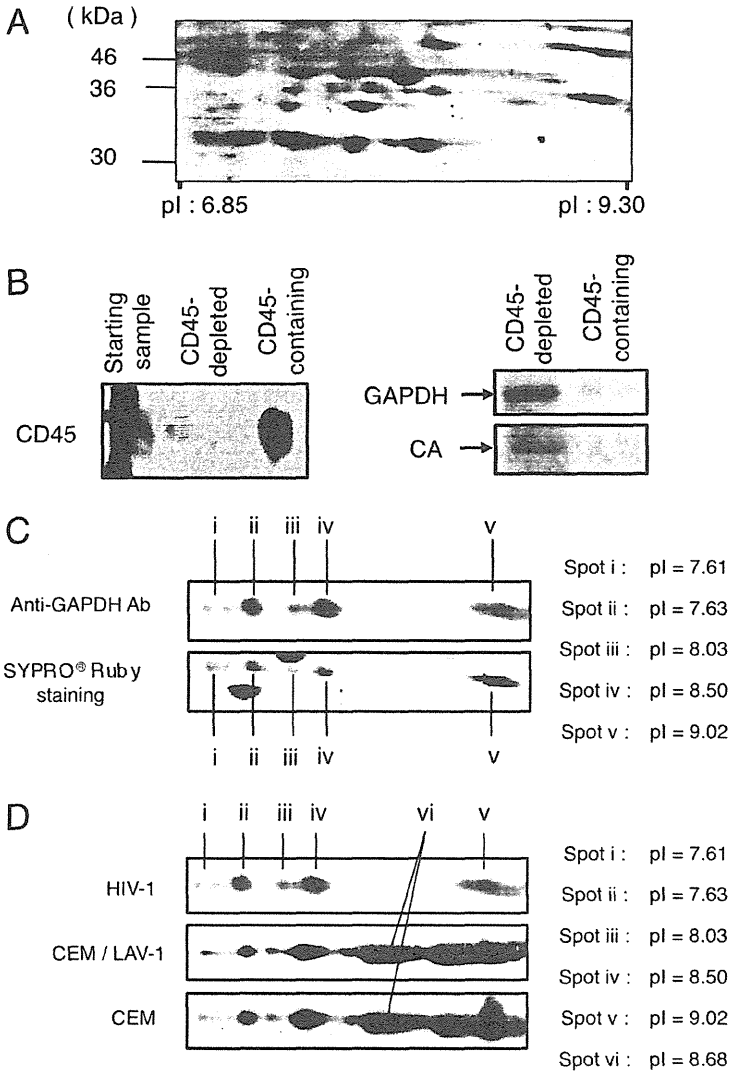

Figure 1 Confirmation of GAPDH incorporation into HIV-1 virions. (**A**) SYPRO® Ruby-stained 2D gel image of proteins from highly purified HIV-1_{LAV-1} virions within the pI range of 6.85-9.30 and the molecular weight range of 30–46 kDa. (**B**) Western immunoblotting of GAPDH in CD45-depleted fraction and CD45-containing fraction. CD45 and CA levels were used as the markers of microvesicles and viral particles, respectively. Samples from the CD45-immunoaffinity-depleted fraction (CD45-depleted fraction) and anti-CD45 beads fraction (CD45-containing fraction) are identified above their respective lanes. The antibodies used are indicated on the left side of each blot. (**C**) Detection of virion-associated GAPDH isozymes. The upper panel shows the results of western immunoblotting using an anti-GAPDH antibody and the lower panel indicates the corresponding SYPRO® Ruby-stained gel. (**D**) Comparison of pI values of GAPDH isozymes among HIV-1_{LAV-1} (upper panel), CEM/LAV-1 (middle panel), and CEM cells (lower panel). GAPDH isozymes were detected by western immunoblotting using the anti-GAPDH antibody. Spot pI values are shown in the figure.

packaging inside the virions enhances viral infection owing to the high reverse transcription efficiency. These results elucidate the viral replication capacity gained by GAPDH packaging and reveal a novel regulation step of HIV-1 infection.

Results

Multiple GAPDH isozymes were incorporated into virions

To examine the host proteins found in virions produced by an HIV-infected cell line, CEM/LAV-1 cells, a highly purified HIV-1_{LAV-1} preparation was analyzed by 2D gel electrophoresis, followed by MALDI-TOF MS/MS (Figure 1A). The MS/MS ion search predicted the identity of the 36-kDa proteins to be GAPDH. To confirm whether GAPDH is incorporated into virions, contaminating particles such as microvesicles and exosomes were removed according to previously described methods [19]. Western immunoblotting for CD45, GAPDH, and the capsid (CA) protein was carried out. In the starting sample, the CD45 signal was detected (Figure 1B left panel). On the other hand, no CD45 signal was detected in the lane of the CD45-depleted fraction (flow-through fraction), indicating that most of the CD45-positive contaminating particles such as microvesicles and exosomes in the starting material were removed (Figure 1B left panel). Therefore, as shown in Figure 1B right panel, western immunoblotting for GAPDH, and the CA protein was carried out. The data showed that there were evident GAPDH and CA signals in the CD45-depleted fraction, while there is hardly any GAPDH signal in the CD45-containing fraction, indicating that GAPDH from CEM/LAV-1 cells may exclusively be incorporated into the virions (Figure 1B right panel). Therefore, unless otherwise noted, HIV-1 preparations were systematically prepared without the CD45 depletion procedure in the following study. To gain further insight into the incorporation of GAPDH into virons, we analyzed GAPDH incorporated into virions and present in CEM/LAV-1 cells and the HIV-1-noninfected cell line CEM cells using 2D gel electrophoresis and western immunoblotting. We found that five isozymes of GAPDH (spot i (pI 7.61), spot ii (pI 7.63), spot iii (pI 8.03), spot iv (pI 8.50), and spot v (pI 9.02)) were inside the virions (Figure 1C). Surprisingly, regardless of HIV-1 infection, in the cellular pool, GAPDH was identified as six isozymes that contain an additional isozyme (spot vi (pI 8.68)) with five isozymes packaged inside the virions (Figure 1D). These results suggest that the GAPDH isozymes (pI 7.61, 7.63, 8.03, 8.50, and 9.02) might be selectively packaged inside the virions. Therefore, we focused primarily on the characterization of GAPDH present within the virions.

GAPDH-packaging-defective virus exhibits higher infectivity than control viruses

To determine whether a decrease in the packaging level of virion-associated GAPDH affects HIV-1 infectivity, we transfected CEM/LAV-1 cells with GAPDH siRNA or control siRNA, and observed no toxic effects on cell viability and proliferation (Figure 2A). As shown in Figures 2B and C, GAPDH siRNA specifically decreased GAPDH level not only in CEM/LAV-1 cells without altering the level of actin but also in the virions without altering the levels of gp120, HIV-1 RT, $Pr55^{gag}$, and CA protein. Furthermore, p24 ELISA indicated that the GAPDH-packaging-defective virus did not show a defect in viral release (Figure 2D) and the incorporation of HIV genomic RNA into viral particles (Figure 2E). However, the GAPDH-packaging-defective virus showed an increased infectivity (Figure 2F, $p< 0.01$) compared with the control virus. To further clarify the relationship between the increased infectivity and the reverse transcription efficiency of the GAPDH-packaging-defective virus in TZM-bl cells, we carried out quantitative real-time PCR analysis to quantify the early strong-stop DNA of reverse transcription. The GAPDH-packaging-defective virus showed a significant increase in the level of the early strong-stop form of the viral cDNA product (Figure 2G, $p<0.01$). These results suggest that the enhanced infectivity of the GAPDH-packaging-defective virus is linked to a higher efficiency of the early reverse transcription process.

A suppressed packaging of GAPDH is accompanied by an enhanced packaging of LysRS and $tRNA^{Lys3}$ in the virions

To investigate why the GAPDH-packaging-defective virus showed a significant increase in the level of the early (R/U5) form of the viral cDNA product, *in vitro* HIV-1 RT activity was quantified in the presence of GAPDH. In RT assay, the ability of HIV-1 RT to synthesize DNA is utilized using hybrid poly(A)-oligo $(dT)_{15}$ as a template and the primer, in the presence of recombinant GAPDH. The assays showed that GAPDH did not allosterically inhibit the activity of HIV-1 RT (Figure 3A). Gabor *et al.* [4] reported that a greater packaging of LysRS into virions is accompanied by increased $tRNA^{Lys3}$ packaging, initiation of reverse transcription, and increased infectivity of the viral population. Therefore, we next investigated whether a decrease in virion-associated GAPDH level results in an enhanced packaging of LysRS and $tRNA^{Lys3}$ inside the virions. Figures 3B and C show the effect of GAPDH siRNA treatment on the packaging of LysRS and $tRNA^{Lys3}$ inside the virions. Western immunoblotting and quantitative real-time PCR analysis demonstrated that the reduction in virion-associated GAPDH level correlates with an about 1.5-fold increase in viral LysRS packaging

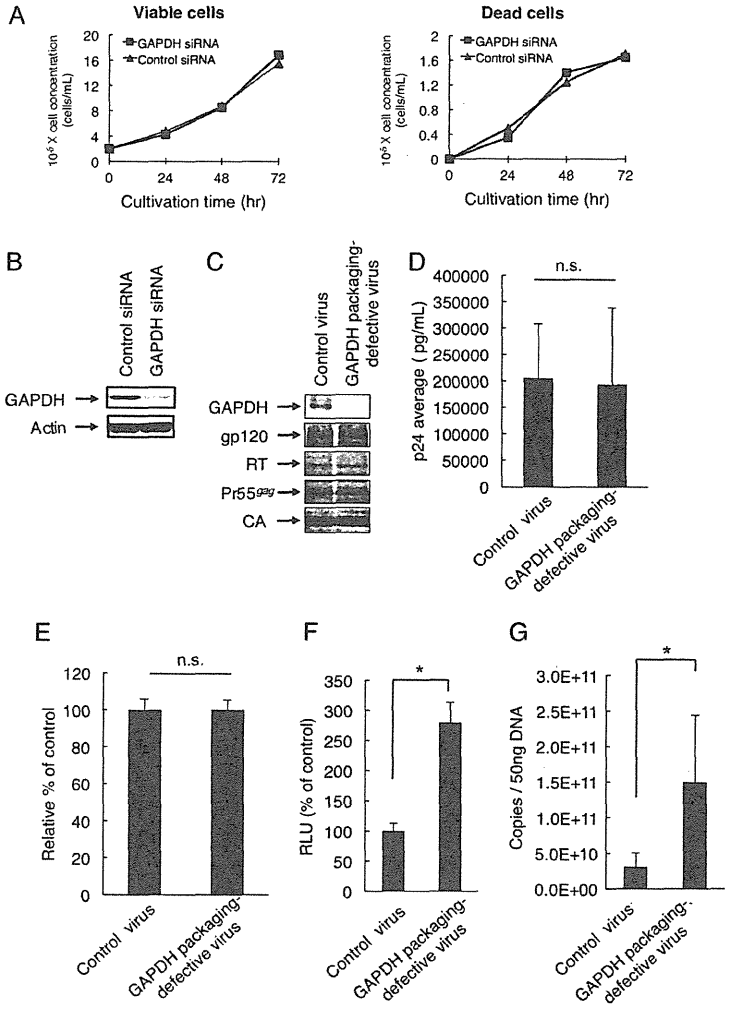

Figure 2 Effects of GAPDH packaging defect inside virions on HIV-1 replication. (**A**) Effects of GAPDH siRNA treatment on CEM/LAV-1 cells. Viable cells (left) and dead cells (right) were assessed by trypan blue staining. (**B**) GAPDH siRNA knockdown efficiency is confirmed by western immunoblotting. Expression of GAPDH was analyzed in cell lysates from CEM/LAV-1 cells transfected with GAPDH or control siRNA 72 h after transfection. (**C**) Effects of GAPDH siRNA on incorporation of GAPDH and HIV-1 proteins inside virions. The incorporation of GAPDH, gp120, RT, $Pr55^{gag}$, or CA was analyzed by western immunoblotting of lysates from viruses produced from CEM/LAV-1 cells transfected with GAPDH or control siRNA. The anti-GAPDH antibody and HIV-1-positive plasma were used for western immunoblotting. (**D**) Effects of GAPDH siRNA on virus release in CEM/LAV-1 cells transfected with GAPDH or control siRNA. The virus release in culture supernatant was directly determined by p24 ELISA. The mean values of at least three independent experiments are shown. (**E**) Effects of GAPDH siRNA on the incorporation of HIV genomic RNA into viral particles. The level of genomic RNA in the control virus (normalized to RT activities) was set as 100%. (**F**) Infectivity of GAPDH-packaging-defective virus. Infectivity was evaluated on the basis of the luciferase activity in lysates of TZM-bl cells. The value in the control experiment was set as 100%. The mean values of at least three independent experiments are shown. (**G**) Effect of defect in GAPDH packaging on reverse transcription in TZM-bl cells. The early strong-stop DNA products were determined by quantitative real-time PCR analysis as described in "Methods". The significance of difference (Student's *t-test*) is indicated as follows: *, $p<0.01$; n.s., not significant. The mean values of at least three independent experiments are shown. The *error bars* denote the standard deviation.

level (Figure 3B) and an about 4-fold increase in viral $tRNA^{Lys3}$ packaging level (Figure 3C, $p< 0.01$). Because Javanbakht *et al.* [20] and Mak *et al.* [21] further reported that LysRS packaging requires the viral $Pr55^{gag}$ and $tRNA^{Lys3}$ packaging additionally requires the $p160^{gag\text{-}pol}$ precursor, we examined whether GAPDH could interact directly with $Pr55^{gag}$ and $p160^{gag\text{-}pol}$. Immunoprecipitation assay using an anti-GAPDH antibody showed that GAPDH directly interacts with $Pr55^{gag}$ and $p160^{gag\text{-}pol}$ (Figure 3D). Furthermore, we concluded that GAPDH does not directly interact with LysRS because no sufficient signal was detected (data not shown). However, the overexpression of LysRS in HIV-1-producing cells results in an increase in LysRS

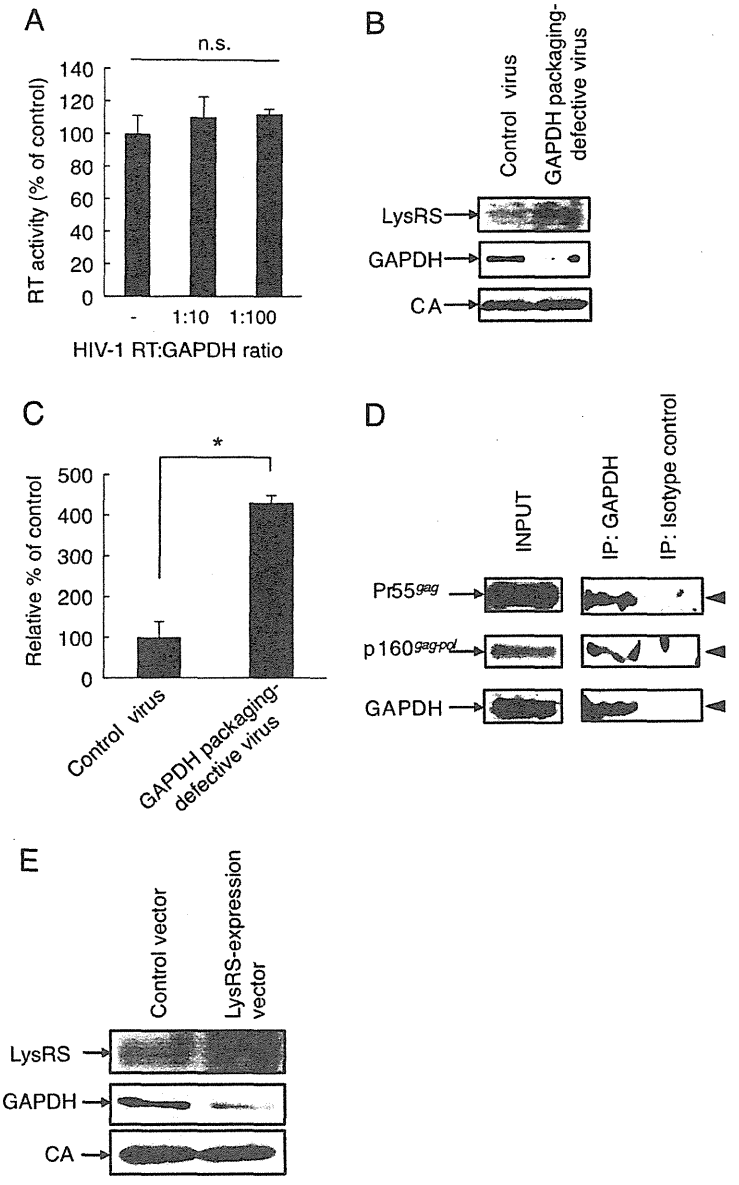

Figure 3 Packaging of LysRS and tRNA^{Lys3} is regulated by GAPDH. (**A**) Effect of GAPDH on enzymatic activity of HIV-1 RT. RT activity assay was performed as described in "Methods". The value in the control experiment was set as 100%. The activity in the presence of GAPDH (RT: GAPDH ratio= 1:10 or 1:100) is shown as the activity relative to that of the control. The mean values of at least three independent experiments are shown. Packaging of (**B**) LysRS and (**C**) tRNA^{Lys3} in GAPDH-packaging-defective virus. (**B**) LysRS and GAPDH were detected by western immunoblotting using anti-LysRS and anti-GAPDH antibodies in lysates from viral particles produced from CEM/LAV-1 cells transfected with GAPDH or control siRNA. (**C**) Incorporated tRNA^{Lys3} level was determined by reverse transcription quantitative PCR analysis as described in "Methods" and normalized by viral genomic RNA level. The amount of tRNA^{Lys3} in the control virus was set as 100%. The mean values of at least three independent experiments are shown. (**D**) Interaction of GAPDH with Pr55^{gag} and p160$^{gag\text{-}pol}$. GAPDH was immunoprecipitated from the clarified lysate from CEM/LAV-1 cells with the anti-GAPDH antibody. The precipitated proteins were analyzed by western immunoblotting using the indicated antibodies to the proteins shown (anti-p24 antibody for Pr55^{gag} and anti-RT antibody for p160$^{gag\text{-}pol}$), and were visualized by enhanced chemiluminescence analysis. The significance of difference (Student's *t-test*) is indicated as follows: *, $p<0.01$; n.s., not significant. The *error bars* denote the standard deviation. (**E**) The packaging of GAPDH in the enhanced-LysRS-packaging virus. The packaging efficiencies of LysRS and GAPDH were analyzed in lysates from viral particles produced from HEK293 cells cotransfected with pNL-CH and either the LysRS expression or control vector.

packaging level in HIV particles but a decrease in GAPDH packaging level (Figure 3E). These results suggest that the incorporation of GAPDH inside virions may contribute to the suppression of initiation of reverse transcription owing to the suppression of the packaging of LysRS and $tRNA^{Lys3}$ inside the virions.

An enhanced GAPDH packaging decreased viral infectivity

We next investigated whether the virus produced from HEK293 cells with high GAPDH expression levels showed decreased infectivity. We cotransfected HEK293 cells with the HIV-1 expression plasmid pNL-CH and either a GAPDH expression vector or a control vector. As shown in Figure 4A, the GAPDH expression vector increased the GAPDH level inside the virions. As expected, the enhanced-GAPDH-packaging virus decreased the viral infectivity in TZM-bl cells because the enhanced GAPDH packaging decreased $tRNA^{Lys3}$ packaging (Figures 4B and C). Furthermore, we examined whether the enhanced packaging of GAPDH suppressed the viral replication in primary PBMCs.

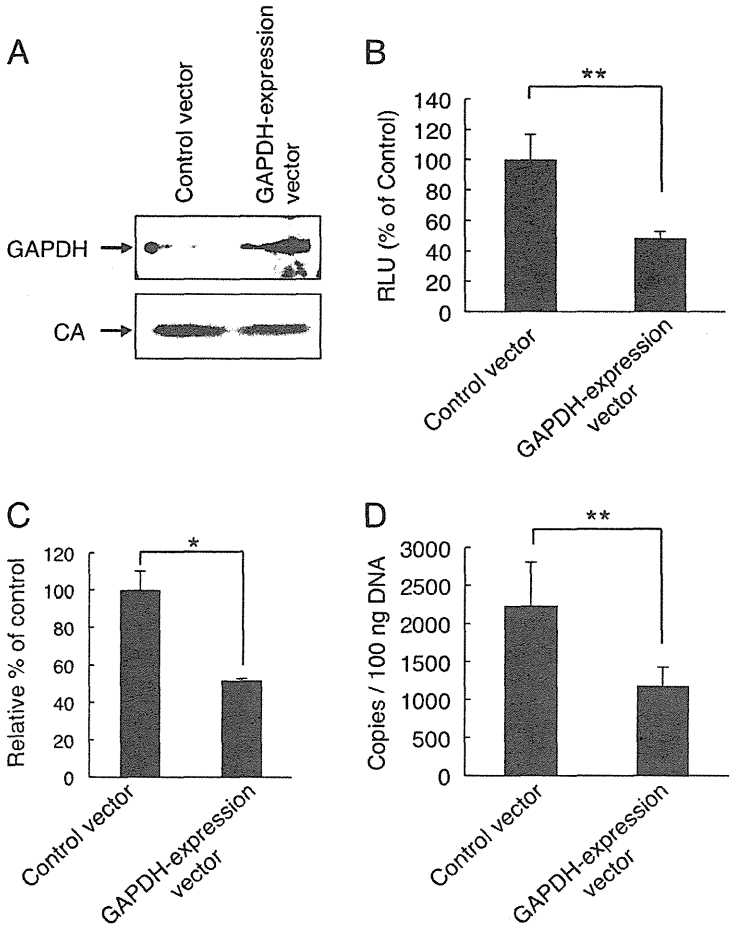

Figure 4 Effects of enhanced GAPDH packaging inside virions on HIV-1 replication. (**A**) Enhanced GAPDH packaging inside virions. The packaging efficiency of GAPDH was analyzed in lysates from viral particles produced from HEK293 cells cotransfected with pNL-CH and either the GAPDH expression or control vector. (**B**) Infectivity of enhanced-GAPDH-packaging virus. TZM-bl cells were incubated with an equal amount (2 ng of p24 antigen) of the enhanced-GAPDH-packaging virus or the control virus. Infectivity was evaluated as described in "Methods". The mean values of at least three independent experiments are shown. (**C**) Quantification of $tRNA^{Lys3}$ in enhanced-GAPDH-packaging virus. Incorporated $tRNA^{Lys3}$ level was determined by reverse transcription quantitative PCR analysis as described in "Methods" and normalized by viral genomic RNA level. The amount of $tRNA^{Lys3}$ in the control virus was set as 100%. The mean values of at least three independent experiments are shown. (**D**) Enhanced GAPDH packaging inside virions decreased reverse transcription efficiency in PMBCs. PBMCs were incubated with an equal amount (20 ng of p24 antigen) of the enhanced-GAPDH-packaging virus or the control virus. Reverse transcription products (R/gag DNA products) were determined as described in "Methods". The significance of difference (Student's *t-test*) is indicated as follows: **, $p<0.05$. The mean values of at least three independent experiments are shown. The *error bars* denote the standard deviation.

Interestingly, the enhanced-GAPDH-packaging virus showed a significant decrease in the level of the R/gag DNA product in PBMCs (Figure 4D). These results suggest that an enhanced GAPDH packaging decreased viral infectivity.

Discussion

Current drugs against HIV-1 inhibit the function of viral enzymes, namely, RT, integrase, and protease. While these are especially proving useful in combination therapy, drug resistance could still be generated and appears to result from viral replication that allows the error-prone reverse transcription step. Therefore, future directions towards a more effective therapy for HIV-1 infection will rely on the development of novel therapeutic strategies rather than conventional strategies, which target only viral proteins. HIV-1 exploits multiple host proteins during infection, suggesting that the possibility of interrupting virus-host interactions may be an important pathway for the development of antiviral therapies. In concept, this type of antiviral agent developed through this pathway could minimize the generation of drug-resistant mutants because the virus must maintain the ability to interact with the relatively immutable host proteins. Thus, the interaction between the host and viral proteins may offer some potential applications for therapies against HIV-1.

Proteomic analysis of HIV-1 particles is a powerful tool to identify not only novel host proteins that are packaged during viral budding but also post-translational modifications of host and viral proteins in the virions. In our previous study, three isoforms of cyclophilin A associated with HIV-1 and the post-translational modifications such as *N*-acetylation of cyclophilin A and formylation of CA protein were identified [22,23]. In this study, GAPDH isozymes with different isoelectric points were detected in the virions. Although Chertova *et al.* [24] and Ott *et al.* [25] also reported the GAPDH incorporation into HIV-1 virions produced from lymphocytes and macrophages, the virological significance of GAPDH has remained unclear. To gain insight into the role of GAPDH in viral replication, we prepared the GAPDH-packaging-defective virus by suppressing GAPDH expression by RNA interference in CEM/LAV-1 cells, and investigated the phenotypic and functional property changes of the virus. Here, we demonstrated that the suppression of GAPDH packaging increased the efficiencies of LysRS and $tRNA^{Lys3}$ packaging into the virions, resulting in the increase in infectivity, and that GAPDH directly interacted with $Pr55^{gag}$ and $p160^{gag\text{-}pol}$, which are required for the packaging of the LysRS and $tRNA^{Lys3}$ complex. Javanbakht *et al.* [20,26] reported that the interaction between the *gag* protein and LysRS is dependent on the last 54 amino acids of the CA C-terminal domain of the *gag* protein and amino acids 208–259 of LysRS and that the packaging of $tRNA^{Lys3}$ requires interaction with LysRS. Furthermore, Khorchid *et al.* [27] reported that the interaction between $p160^{gag\text{-}pol}$ and $tRNA^{Lys3}$ involves the thumb domain (TH) sequences in RT. From these findings, Kleiman *et al.* postulated a conventional model for the formation of a $tRNA^{Lys3}$ packaging complex, in which a $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral genomic RNA complex interacts with a $tRNA^{Lys3}$/LysRS complex. On the other hand, our findings suggest that GAPDH and $tRNA^{Lys3}$/LysRS complex may compete with each other for the interaction to the binding domain within the $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral genomic RNA complex (Figure 5). Taken together, these results indicate that GAPDH negatively regulates HIV-1 infection, and small molecules that reconstitute the binding mode of GAPDH to $Pr55^{gag}$ and $p160^{gag\text{-}pol}$ may interrupt $Pr55^{gag}$-LysRS or $p160^{gag\text{-}pol}$-LysRS interactions.

Conclusions

We conclude that GAPDH is incorporated into virions and that this negatively regulates HIV-1 infection by reducing LysRS and $tRNA^{Lys3}$ packaging efficiency. Elucidating the role of host proteins in HIV-1 infection is a critical issue and may reveal novel mechanisms of pathogenesis that may lead to the discovery of new antiviral targets.

Methods

Cell culture

A chronically HIV-$1_{LAV\text{-}1}$-infected T-cell line (CEM/LAV-1) and HEK293 cells were maintained at 37°C in RPMI-1640 and DMEM supplemented with 10% fetal calf serum (FCS) containing 100 IU/ml penicillin and 100 μg/ml streptomycin in 5% CO_2. TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. This is a HeLa cell clone that was engineered to express CD4 and CCR5 and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under the control of an HIV-1 LTR, permitting sensitive and accurate measurements of infection. PBMCs were isolated using Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. For concanavalin A (ConA) stimulation of PBMCs, 2×10^6 cells were incubated for 24 h in RPMI-1640 containing 10% FCS, 4 mM l-glutamine, 2 mM sodium pyruvate, 50 μM 2-ME, and 25 μg/mL ConA (Sigma, St. Louis, MO). After stimulation with ConA, PBMCs were further incubated for 72 h in RPMI-1640 containing 10% FCS, 4 mM l-glutamine, 2 mM sodium pyruvate, 50 μM 2-ME, and 100 U/ml IL-2.

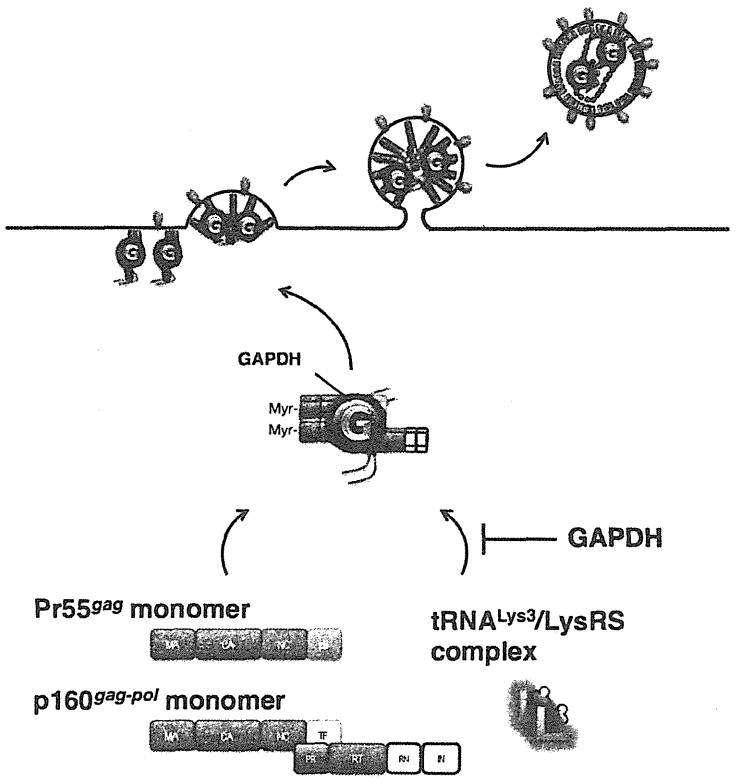

Figure 5 Schematic illustration showing the mechanism by which GAPDH negatively regulates HIV-1 infection. A single copy of each molecule is shown for simplicity. As described in detail elsewhere [20,26,27], a $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral RNA complex interacts with a $tRNA^{Lys3}$/LysRS complex. Although the actual conformation of the $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral RNA complex is still unclear, findings of this study suggest that GAPDH and $tRNA^{Lys3}$/LysRS complex may compete with each other for interaction with the binding site within $Pr55^{gag}$ and p160gag-pol.

Plasmids

The coding regions of human GAPDH cDNAs (GenBank™ accession number M33197.1) were amplified by PCR using the primers GF (5′-AGGATCCGCCATGGGGAAGGTGA AGGTCGG-3′) and GR (5′-GCCCACATGGCCTCCAA GGAGGATATCA-3′). The regions were cloned into the *Eco*RV and *Bam*HI sites of the pcDNA™ 3.1D/V5-His-TOPO® vector (Life Technologies Corporation). The coding regions of the human LysRS cDNAs (GenBank™ accession number NM_001130089) were amplified by PCR using the primers LF (5′-AGGTACCGCCATGTTGAC GCAAGCTGCTGTAAGG-3′) and LR (5′-ATTCGAAGA CAGAAGTGCCAACTGTTG-3′). The regions were cloned into the *Kpn*I and *Bst*BI sites of the pcDNA™ 3.1D/V5-His-TOPO® vector (Life Technologies Corporation).

Viruses

Infectious $HIV\text{-}1_{LAV\text{-}1}$ stocks were prepared from culture supernatants of CEM/LAV-1 cells [28]. Briefly, the supernatant from the culture medium of CEM/LAV-1 cells was filtered through a 0.22-μm-pore size disposable filter and then centrifuged at 43,000 × *g* for 3 h at 4°C. The obtained pellet was resuspended in PBS(−) (0.02% KH_2PO_4, 0.29% $Na_2HPO_4{\cdot}12H_2O$, 0.8% NaCl, 0.02% KCl) and then centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting pellet was resuspended in PBS(−) and used as an infectious $HIV\text{-}1_{LAV\text{-}1}$ stock. GAPDH-packaging-defective viruses were prepared by GAPDH siRNA treatment of CEM/LAV-1 cells. CEM/LAV-1 cells (5×10^5 cells) were transfected with 50 nM validated commercially available Silencer GAPDH siRNA (Catalog #: AM4605, the siRNA sequence is not released, Life Technologies Corporation) or 50 nM control siRNA. After 24 h, the CEM/LAV-1 cells were washed and cultured for 48 h with antibiotic-free RPMI containing 10% FCS. The virus-containing supernatant was collected 72 h after transfection. On the other hand, the enhanced-GAPDH- or enhanced-LysRS-packaging virus was prepared by cotransfection of HEK293 cells (5×10^5 cells) with pNL-CH [29] and either the pcDNA™ 3.1D-GAPDH-V5-His-TOPO® (GAPDH expression vector) or pcDNA™ 3.1D-LysRS-V5-His-TOPO® (LysRS expression vector). pNL-CH, derived from the pNL4-3 clone of

HIV-1, contains a silent T-to-C mutation at nucleotide 2600 to introduce an *Rsr*II restriction enzyme site near the 5′ end of *pol*.

CD45 affinity depletion

HIV-1_{LAV-1} was collected from CEM/LAV-1 culture medium and prepared according to previously described methods [28], with slight modifications. The supernatant from the culture medium of CEM/LAV-1 cells was filtered through a 0.22-μm-pore size disposable filter and then centrifuged at 43,000 × *g* for 3 h at 4°C. The obtained pellet was resuspended in PBS(−) and then centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting pellet was resuspended in PBS(−). To remove CD45-positive contaminating particles such as microvesicles and exosomes from HIV-1 preparations, HIV-1 preparations containing microvesicles and exosomes were incubated with 2 μl of prewashed CD45 microbeads (Miltenyi Biotech, Auburn, CA) per 1 μg of total protein overnight on ice according to previously described methods [19], with slight modifications. In Figure 1B, forty micrograms (total protein) of purified HIV-1_{LAV-1} produced from CEM/LAV-1 cells (starting sample) was subjected to the CD45 depletion procedure. The mixture of sample-CD45 microbeads was then applied to a washed and magnetized Macs separation column (Miltenyi Biotech). The flow-through fraction was collected to obtain the CD45-depleted fraction. The column was sufficiently washed with PBS(−). To recover the contaminating particles retained on the beads as the CD45-containing fraction, preheated (95°C) SDS gel loading buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol (pH 6.8), and 10% 2-mercaptoethanol) was applied on to the column matrix. To compare the CD45 level between the "CD45-depleted" and "CD45-containing" lanes in Figure 1B, equal amounts of samples by volume were loaded on the gel in accordance with the method of Ott *et al.* (*J. Virol.* 2003, 77:12699–12709.). Twenty microliter portions of fractions equivalent to the starting sample (by volume) were analyzed by CD45 immunoblotting (Figure 1B).

Two-dimensional electrophoresis analysis

For 2D gel electrophoresis, the viral pellets derived from HIV-1_{LAV-1} were boiled for 5 min and lysed with 100 μl of lysis buffer (9.5 M urea, 2% (w/v) NP-40, 2% ampholine (pHs 3–10), and 5% 2-mercaptoethanol). Furthermore, CEM/LAV-1 or CEM cells were also lysed with the same lysis buffer. An aliquot of the lysate (1 mg of proteins for in-gel digestion, or 300 μg of proteins for western immunoblotting) was subjected to 2D polyacrylamide gel electrophoresis according to the method of O'Farrell [30].

Protein identification by mass spectrometry

The gels obtained after 2D gel electrophoresis were stained with SYPRO® Ruby (Invitrogen). SYPRO® Ruby-stained gel pieces were excised from the gel and in-gel-digested with trypsin, as previously described [28]. For the analysis in the MS and MS/MS mode, 1 μl of the matrix (alpha-cyano-4-cinnamic acid saturated in 40% acetonitrile, 0.1% trifluoroacetic acid) per microliter of the tryptic digest was deposited on a ground steel target plate. Analysis was conducted using a MALDI-TOF/TOF UltrafleXtreme (Bruker Daltonics Inc.). Database search was performed on the NCBInr database using Mascot software (Matrix Science, London, UK).

One-dimensional electrophoresis and western immunoblotting

SDS-polyacrylamide gel electrophoresis was performed on 5-20% polyacrylamide gels according to the method of Laemmli [31]. Electrophoresis was carried out at 20 mA per gel for 90 min. Proteins were then transferred onto a PVDF membrane at 0.8 mA/cm^2 for 70 min. The membrane was saturated in 5% skimmed milk powder in TBS (10 mM Tris–HCl (pH 7.5), 0.5 M NaCl) and incubated for 4 h with each primary antibody. After washing in TBS-T (10 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 0.5% Tween 20), the membrane was further incubated for 1 h with each secondary antibody, and immunoreaction products were visualized using a SuperSignal® West Pico Chemiluminescent substrate (Thermo Fisher Scientific Inc.).

Suppression of GAPDH packaging by siRNA and virus release assay

To examine the cytotoxicity induced by siRNA treatment, trypan blue dye exclusion assay was carried out. GAPDH siRNA knockdown efficiency in CEM/LAV-1 cells, which were transfected with GAPDH or control siRNA, 72 h after transfection was confirmed by western immunoblotting using an anti-GAPDH antibody (SIGMA). The packaging efficiency of GAPDH, LysRS, and viral structural proteins into virions was monitored by western immunoblotting using an anti-GAPDH antibody, an anti-LysRS antibody (Cell Signaling Technology), and HIV-1-positive plasma (a kind gift from Dr. Shuzo Matsusita, AIDS Research Institute, Kumamoto University, Kumamoto, Japan). The release of the GAPDH-packaging-defective virus was directly monitored by p24 ELISA (ZeptoMetrix Corporation) as the amount of the CA protein in culture supernatants of CEM/LAV-1 cells transfected with GAPDH or control siRNA.

Determination of HIV-1 infectivity

The infectivity of the GAPDH-packaging-defective virus or enhanced-GAPDH-packaging virus was quantified

using TZM-bl cells, which express a luciferase gene and a beta-galactosidase gene under the control of the HIV-1 LTR promoter [32]. TZM-bl cells (4×10^5 cells) were incubated with the GAPDH-packaging-defective, enhanced-GAPDH-packaging, or control virus with 20 μg/ml DEAE dextran for 2 h at 37°C and then cultured in DMEM supplemented with 10% FCS (200 μl) for 48 h. The cells were fixed, and the HIV-1 infection of TZM-bl cells was determined by measuring the luciferase activity in cell lysates.

Quantitative analysis of HIV-1 reverse transcription during acute infection

De novo-synthesized HIV-1 cDNA was analyzed using the protocol of Ikeda *et al.* [33]. Briefly, the TZM-bl cells or PBMCs (1×10^6 cells) were infected with either the GAPDH-packaging-defective virus or the enhanced-GAPDH-packaging virus and incubated for 4 h at 37°C. The cells were washed with PBS(−), incubated for 20 h at 37°C, washed with PBS(−), and further incubated for 5 min at 37°C in PBS(−) containing 0.25% trypsin. After trypsinization, the cells were washed twice with PBS(−) and then digested in 200 μl of digestion buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 100 μg/ml proteinase K) for 2 h at 50°C. After digestion, proteinase K was heat-inactivated for 10 min at 95°C. To measure the amounts of early reverse transcription products in GAPDH-packaging-defective virus infection, the sample was subjected to quantitative real-time PCR with a primer pair specific for the R/U5 region (M667, 5′-GGCTAACTAGGGAACCCACTG-3′; AA55: 5′-CTGCTAGAGATTTTCCACACTGAC-3′). To further measure the amounts of late reverse transcription products in enhanced-GAPDH-packaging virus infection, a primer pair specific for the R/gag region (M667, 5′-GGC TAACTAGGGAACCCACTG-3′; M661, 5′-CCTGCGTC GAGAGAGCT CCTCTGG-3′) was used. Because the primer pair R/U5 used detects both early and late products, the following computation was used to determine the amount of early strong-stop DNA: the copy number of strong-stop DNA=R/U5 DNA-R/gag DNA copies.

RT activity assay

To investigate whether GAPDH could allosterically reduce RT activity, recombinant GAPDH (Sigma-Aldrich Co., LLC.) and a reverse transcription assay kit (F. Hoffmann-La Roche Ltd.) were used in this assay. Briefly, the solution (46 mM Tris–HCl, 266 mM potassium chloride, 27.5 mM magnesium chloride, 9.2 mM DTT, digoxigenin (DIG)-labeled dUTP, biotin-labeled dUTP, dTTP, and poly(A) × oligo$(dT)_{15}$ template/primer hybrid) was added to the reaction tube containing the HIV-1 RT standard preincubated for 1 h with recombinant GAPDH at a ratio of 1:10 or 1:100, and then incubated for 1 h at 37°C. After finishing the RT reaction, the reaction mixture was transferred to streptavidin-coated microtitre plates. DIG-labeled DNA was detected with an anti-DIG-POD conjugate, reacted with 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid, and quantitated by measuring OD at 405/490 nm. The HIV-1-RT inhibition assay was performed as described in the kit protocol.

Quantification of viral genomic RNA and $tRNA^{Lys3}$ packaging levels in virions

Both viral genomic RNA and $tRNA^{Lys3}$ were collected using a QIAamp® Viral RNA Mini kit (Qiagen) or Nucleo Spin® miRNA (Macherey-Nagel). Genomic RNA was reverse-transcribed using a SuperScript® VILO™ cDNA Synthesis kit and quantified using a primer pair specific for the R/gag region (M667, 5′-GGCTAA CTAGGGAACCCACTG-3′; M661, 5′-CCTGCGTCGAG AGAGCTCCTCTGG-3′), or the primers SK38 (5′-ATA ATCCACCTATCCCAGTAGGAGAAAT-3′) and SK39 (5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′). On the other hand, $tRNA^{Lys3}$ was reverse-transcribed by a SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies Corporation) using a $tRNA^{Lys3}$-F-primer (5′-TGGCGCCCGAACAGGGAC-3′) and then quantified by a $tRNA^{Lys3}$-F-primer and a $tRNA^{Lys3}$-R-primer (5′-GCATCAGACTTTTAATCTGAGGG-3′). Quantitative real-time PCR was carried out with a SsoFAST™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.); the cycling conditions were 98°C for 2 min, then 98°C for 5 sec, followed by 40 cycles of 15 sec at 60°C.

Coimmunoprecipitation

CEM/LAV-1 cells were lysed in 350 μl of RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100). The insoluble material was pelleted, and the supernatant was used for coimmunoprecipitation. The supernatant was precleaned with Protein-G Sepharose® without any antibody, incubated with 10 μl of an anti-GAPDH antibody (Santa Cruz Biotechnology, INC) or an isotype control goat IgG antibody (Southern Biotechnology Associates, Inc.) for 4 h at 4°C, and further incubated with 50 μl of Protein-G Sepharose® resin slurry (50% slurry in RIPA buffer) for 4 h at 4°C. After washing with RIPA buffer, the bound proteins were eluted using the SDS gel loading buffer. The precipitated proteins were detected by western immunoblotting. $Pr55^{gag}$ and $p160^{gag\text{-}pol}$ were detected using an anti-p24 antibody (ViroGen) and an anti-RT antibody (Bio Academia), respectively.

Enhancement of GAPDH packaging by GAPDH expression vector

Enhanced-GAPDH-packaging viruses were prepared by cotransfection of HEK293 cells with pNL-CH and the GAPDH expression vector. Enhanced GAPDH packaging efficiency in viral particles is confirmed by western immunoblotting using an anti-GAPDH antibody (SIGMA). The signal of the CA protein was used as the loading control.

Enhancement of LysRS packaging by LysRS expression vector

Enhanced-LysRS-packaging viruses were prepared by cotransfection of HEK293 cells with pNL-CH and the LysRS expression vector. Enhanced LysRS packaging efficiency in viral particles is confirmed by western immunoblotting using an anti-LysRS antibody (Cell Signaling Technology). The signal of the CA protein was used as the loading control.

Abbreviations

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HIV-1: Human immunodeficiency virus type 1; RT: Reverse transcriptase; LysRS: Lysyl-tRNA synthetase; PBMCs: Peripheral blood mononuclear cells; MALDI-TOF MS: Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; CA: Capsid; MA: Matrix.

Competing interests

The authors have no conflicting financial interests.

Authors' contributions

SM conceptualized and designed the study, NK, AO, KK, NT, SS, and SM performed the study, and analyzed data; NK and SM wrote and critically read the paper. All the authors reviewed the manuscript and approved the final version.

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Review Article

Retrovirus Entry by Endocytosis and Cathepsin Proteases

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Retroviruses include infectious agents inducing severe diseases in humans and animals. In addition, retroviruses are widely used as tools to transfer genes of interest to target cells. Understanding the entry mechanism of retroviruses contributes to developments of novel therapeutic approaches against retrovirus-induced diseases and efficient exploitation of retroviral vectors. Entry of enveloped viruses into host cell cytoplasm is achieved by fusion between the viral envelope and host cell membranes at either the cell surface or intracellular vesicles. Many animal retroviruses enter host cells through endosomes and require endosome acidification. Ecotropic murine leukemia virus entry requires cathepsin proteases activated by the endosome acidification. CD4-dependent human immunodeficiency virus (HIV) infection is thought to occur via endosomes, but endosome acidification is not necessary for the entry whereas entry of CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, is low pH dependent. There are several controversial results on the retroviral entry pathways. Because endocytosis and endosome acidification are complicatedly controlled by cellular mechanisms, the retrovirus entry pathways may be different in different cell lines.

1. Introduction

Retroviruses include many pathogenic agents in humans and animals. Human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV) induce acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia (ATL), respectively. Murine leukemia viruses (MLVs) are also well-studied among retroviruses because the MLVs are used comparatively as animal models of several human diseases (leukemia, immunodeficiency, and neuropathogenic diseases) and as gene transfer tools. In addition, there are animal retroviruses that are important problems in the livestock industry, such as Visna, equine infectious anemia virus, bovine leukemia virus, and Jaagsiekte sheep retrovirus.

Retroviruses contain envelope membranes consisting of lipid bilayers derived from virus-producing cells. Genomes of simple retroviruses such as MLVs encode three essential elements, gag, pol, and env genes. Complex retroviruses including HIV additionally encode accessory genes whose products regulate the retroviral expression and suppress host antivirus factors [1]. The gag and pol genes encode viral structural proteins and enzymes, respectively. These proteins are synthesized as precursor polyproteins and then are cleaved to mature peptides by a protease encoded by the retroviral pol gene.

Retroviral envelope (Env) glycoprotein encoded by the env gene is also synthesized as a precursor protein and is cleaved to surface (SU) and transmembrane (TM) subunits by a cellular protease [2]. Retroviruses enter host cells by fusion between viral envelope and host cell membrane, following the recognition of cognate cell surface receptors. The SU protein binds to the cell surface receptor protein. The TM protein anchors the SU protein to the surface of viral particles and virus-producing cells by the complex formation of SU and TM. The TM protein mediates the membrane fusion reaction. The entry mechanisms of retroviruses are vigorously studied but are not completely understood. Elucidation of the retrovirus entry machinery

would contribute to the development of new therapeutic approaches for retrovirus-induced diseases.

2. Membrane Fusion by Retroviral Env Glycoprotein

Mechanism of membrane fusion by the retroviral TM proteins is described elsewhere in details [3–7] and is similar to those used by envelope proteins of other enveloped viruses [8, 9]. Briefly, the retroviral entry mechanism is proposed as follows. The TM protein is thought to have hairpin-like structure (Figure 1). The binding of SU with its cognate cell surface receptor induces conformational changes of the TM subunit. The N-terminal hydrophobic domain of the TM subunit called fusion peptide is exposed by the conformational change and inserted into host cell membrane. The TM protein then coverts to a trimer-of-hairpins conformation, and viral envelope and host cell membranes approach and mix. Finally, the fusion pore is formed and expanded to derive the viral core into host cell cytoplasm. This conformational change pathway of the TM protein induces the membrane fusion for the retroviral entry into host cells.

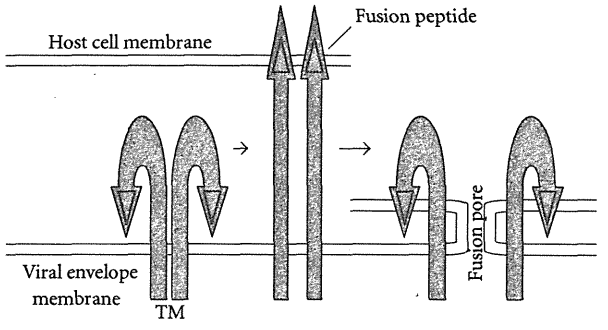

FIGURE 1: Conformational change of retroviral TM subunit for membrane fusion.

3. Retrovirus Receptors

In this section, we will mainly focus on the infection receptors for MLV and HIV, with which entry mechanisms are most extensively studied among retroviruses. Other reviews should be referred to concerning the infection receptors of animal retroviruses in general [10, 11]. MLVs are divided into four groups according to their host ranges and infection interference, and the four groups recognize different cell surface receptors. Ecotropic MLVs infect mouse and rat and bind to cationic amino acid transporter 1 (CAT1) as the infection receptor [12]. Amphotropic MLVs infect many types of mammals, and inorganic phosphate symporter 2 (Pit2) is the amphotropic infection receptor [13, 14]. Polytropic MLVs has a similar host range to the amphotropic MLVs. The amphotropic MLVs cannot infect amphotropic virus-infected cells, because Pit2 are already occupied by the amphotropic Env proteins, called infection interference. Whereas the polytropic MLVs can infect amphotropic virus-infected cells, indicating that the polytropic virus receptor is different from the amphotropic receptor. Polytropic MLVs recognize XPR1 for the infection [15–17], whose physiological function is unknown yet. Xenotropic MLVs recognize the XPR1 as polytropic MLVs, but do not infect mouse cells. These MLV infection receptors are all multimembrane spanning proteins.

The infection receptors of HIV are CD4 and one of chemokine receptors (CXCR4 or CCR5) [18]. However, HIV variants that do not require CD4 for the infection are sometimes isolated from AIDS patients [19, 20] though the infectivity of CD4-independent variants is much lower than that of CD4-dependent viruses [21]. Such CD4-independent HIV variants recognize multimembrane spanning CXCR4 or CCR5 as the sole infection receptor, like the MLVs. CD4 is a single-membrane spanning protein, and HIV variants recognizing CD4 as the sole infection receptor have not been isolated. CD4-independent variants of simian immunodeficiency virus (SIV) are more frequently isolated than CD4-independent HIV variants [22, 23]. It is thought that CD4-independent HIV variants are prototypes of CD4-dependent HIVs [22–24].

4. C-Terminal Tail of Retroviral Env Protein Inhibits Membrane Fusion

When retrovirus-producing and -susceptible cells are mixed, viral Env proteins on the cells can effectively interact with infection receptors on the neighboring susceptible cells via direct cell-to-cell contact. The interactions can have both positive and negative effects on the retrovirus replication. First, they can lead to cell-to-cell infection that allows very rapid and synchronized replication of virus compared to the cell-free infection [25, 26]. This can be advantageous for the virus replication in the presence of antiviral agents [27]. Second, the interactions can induce a negative effect, that is, the rapid apoptotic cell death, via syncytium formation [28–30]. This can be disadvantageous for the virus in that the sustained production of progeny virions becomes impossible. If the apoptotic cell death proceeded more efficiently than the virus replication, it eventually would result in poor progeny virus production. Therefore, it is conceivable that the retroviruses have some mechanisms to attenuate fusion capability of the envelope TM proteins in virus-producing cells and to primarily activate it in retroviral particles upon virion budding. Consistently, such mechanisms have been suggested for the Env TM proteins of MLV and HIV.

In the case of MLV Env protein, C-terminal 16-amino acid peptide of the TM subunit called R peptide is further cleaved by the retroviral protease after the budding [31, 32]. The R peptide-containing Env protein is expressed in the virus-producing cells. The R peptide-truncated MLV Env protein can induce syncytia in susceptible cells, but the R peptide-containing Env protein cannot, indicating that the R peptide negatively regulates the syncytium formation of virus-producing cells [33, 34]. Viral particles carrying the R peptide-containing Env protein have much lower infectivity

Table 1: Inhibitors used in studies of retroviral entry pathway.

Inhibitors	Target
Ammonium chloride	Acidification of intracellular vesicles
Bafilomycin A-l	Acidification of intracellular vesicles
Concanamycin A	Acidification of intracellular vesicles
Dynasore	Dynamin-dependent endocytosis
Chlorpromazine	Clathrin-dependent endocytosis
CA-074Me	Cathepsin B protease
Dynamin DN mutant[1]	Dynamin-dependent endocytosis
Caveolin DN mutant	Caveolin-dependent endocytosis
Clathrin DN mutant	Clathrin-dependent endocytosis
Eps 15 DN mutant	Endocytosis

[1] DN: dominant negative.

than those with the R peptide-cleaved Env, showing that the R peptide cleavage during virion maturation is required for the infectivity [35–37]. It has been reported that the R peptide controls the three-dimensional structure of the SU protein [38] and a disulfide bond between the SU and TM proteins [39], suggesting that the R peptide of TM subunit regulates the receptor-mediated SU conformational changes through the S–S bond between the SU and TM. It has been recently shown that the R peptide-cleaved TM forms separated Env legs, but the R peptide ties the TM legs together [40].

Although the C-terminal domain of the HIV TM protein is not cleaved, it is suggested that interaction between the HIV TM C-terminal region and Gag precursor protein suppresses the membrane fusion activity in virus-producing cells [41]. Processing of the HIV Gag precursor after budding abrogates the suppression of membrane fusion, and the mature virions gain sufficient fusion activity for the entry. The functions of C-terminal tails of retroviral Env proteins to inhibit membrane fusion are conserved among many retroviruses [42–45], though the mechanisms are different. The C-terminal domains of retroviral Env glycoproteins function to maintain the production of progeny virions by suppressing syncytium formation-directed apoptosis of virus-producing cells.

5. PH-Dependent Retrovirus Infection

Ammonium chloride, a weak base, neutralizes acid conditions in intracellular vesicles (Table 1). Concanamycin A and bafilomycin A-1 are specific inhibitors of the ATP-dependent proton pump/vacuolar ATPase (V-ATPase) that serves to acidify endocytic vesicles [46, 47]. To analyze the pH dependence of retrovirus entry, these compounds are frequently used. Additionally these inhibitors may affect trafficking of the intracellular vesicles, because siRNA-mediated knockdowns of subunits of V-ATPase complex affect trafficking of intracellular vesicles [48]. Previously it had been reported that ammonium chloride inhibits ecotropic MLV infection but does not amphotropic and xenotropic MLV infections, showing that ecotropic MLV infection occurs through acidic vesicles, but amphotropic and xenotropic MLV infections do not [49, 50] (Table 2). The more specific inhibitors of endosome acidification (concanamycin A and bafilomycin A-1) suppress all of ecotropic, amphotropic, polytropic, and xenotropic MLV infections [51, 52]. At present, it is generally accepted that ecotropic MLV infection requires acidification, because all the studies consistently reported the suppression of ecotropic virus replication with the inhibitors of endosome acidification. In contrast, it has been shown that xenotropic MLV infections are not suppressed by bafilomycin A-1 [53] (Table 2). Due to the controversial results, the entry pathway of xenotropic MLV is not clear yet. Because different cell lines were used in those reports, the low pH requirement of the xenotropic MLV infection may be dependent on the used cell lines (see below).

In case of avian leukosis virus (ALV) infection, there are also several controversial reports. The earlier reports show that ammonium chloride and bafilomycin do not affect ALV infection, suggesting that ALV infection does not require the acidification [54, 55]. In contrast, it has been recently reported that lowering the pH results in quick and extensive cell-cell fusion by ALV [56] and that the acidification inhibitors suppress ALV infection [57, 58]. It is now thought that receptor binding of ALV induces the Env protein to convert to its prehairpin intermediate at neutral pH [59, 60], and then endosome acidification triggers the formation of the final fusion-active form of the Env protein [61–63]. It has been proposed that the discrepancy came from unusual stability of the Env prehairpin intermediate, consequent ability of fusion to proceed upon washout of the acidification inhibitors after several hours, and the relatively high pH requirement for the outer leaflet mixing [64]. Finally, it is considered that ALV entry requires endosome acidification.

The acidification inhibitors suppress infections by mouse mammary tumor virus (MMTV) [65], foamy virus [66], equine infectious anemia virus (EIAV) [67, 68], Jaagsiekte sheep retrovirus (JSRV) [69], and enzootic nasal tumor virus [70]. These results suggest that infections by many animal retroviruses are low pH dependent.

6. Internalization Pathways

The requirement of low pH for the retrovirus infections reveals that retrovirus particles are internalized into acidic intracellular compartments during virus replication. There are several different pathways for the internalization of molecules; (i) phagocytosis, (ii) macropinocytosis, (iii) clathrin- and dynamin-dependent endocytosis, (iv) caveolin- and dynamin-dependent endocytosis, (v) lipid raft- and dynamin-dependent endocytosis, (vi) clathrin-, caveolin-, and dynamin-independent endocytosis that requires lipid raft, and (vii) dynamin-, clathrin-, caveolin-, and lipid raft-independent endocytosis [48, 71]. Here we will briefly summarize the accepted mechanisms and roles of internalization, relevant to the present review [48, 72, 73].

6.1. Phagocytosis. Specialized cells such as macrophages, neutrophils, and monocytes clear debris and pathogens

Table 2: Differential dependence of HIV and MLV infections on endosome acidification.

Viruses	Dependence of acidification	Cell lines	Reference
Ecotropic MLV	Independent	Rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[49, 51, 52]
Amphotropic MLV	Independent	Mouse NIH3T3, rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[51, 52]
Polytropic MLV	Independent	Rat XC	[52]
	Dependent	Mouse NIH3T3, human RE671, rat XC	[52]
Xenotropic MLV	Independent	Human HT1080, HTX, porcine, rat XC	[49, 50, 52, 53]
	Dependent	Mouse NIH3T3, human RE671	[52]
CD4-dependent HIV	Independent	Human CEM, HeLa, C8166, VB	[49, 89–93]
	Independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dependent	Human 293T, HeLa, TE671	[21]

by phagocytosis. Signaling cascades induce the actin rearrangement and form membrane extensions that cover the target particles and engulf it. Phagosomes become acidic by fusion with lysosomes (pH 5.0-6.0). Debris internalized by phagocytosis is degraded in the acidic phagosomes (phagolysosomes).

6.2. Macropinocytosis. Stimulation by certain growth factors or other signals causes membrane protrusions that fuse with the plasma membrane to form large intracellular vesicles known as macropinosomes that encapsulate large volumes of the extracellular fluid. Macropinosomes can either fuse with lysosomes (pH 5.0-6.0) or recycle back to the cell surface. There is no consensus as to the final fate of macropinosomes. Trafficking of macropinosomes seems to depend on cell type and mode of macropinocytosis induction.

6.3. Clathrin-Mediated Endocytosis. After ligands bind to their receptors, the receptor proteins are internalized into intracellular vesicles called endosomes. The endosome formation requires dynamin GTPase, and the endosomes are coated by clathrin proteins. Many receptors are segregated from their ligands in early endosomes due to weakly acidic condition (pH 6.0). Early endosomes become more acidic by V-ATPase-mediated acidification (late endosomes/lysosomes) (pH 5.0-6.0), and separated ligands are degraded by endosome proteases. Certain receptors are transferred from early endosomes to recycling endosomes (pH 6.4) and are reused on the plasma membrane. Some proteins are also recycled from late endosomes/lysosomes through the trans-Golgi network. Lysosomes often form multivesicular bodies.

6.4. Caveolin-Mediated Endocytosis. Glycosylphosphatidylinositol (GPI)-anchored proteins, simian virus 40 (SV40), and cholera toxin trigger the formation of caveolae coated by caveolin proteins. These ligands are internalized into intracellular vesicles (pH 7.0) dependently on dynamin GTPase. The vesicles can be sorted to endosomes and become acidic.

6.5. Clathrin- and Caveolin-Independent Endocytosis. Cholera toxin and SV40 can also be internalized via raft microdomains into GPI-anchored protein-enriched endosomes. Mechanisms regulating this internalization pathway are unclear as of yet.

7. Internalization of Retroviral Particles into Intracellular Vesicles

A dominant negative mutant of caveolin [74], siRNA-mediated knockdown of dynamin, and a dynamin inhibitor (dynasore) (Table 1) [52] suppress the amphotropic MLV infection, suggesting that amphotropic MLV particles are internalized by the dynamin- and caveolin-dependent endocytosis for productive infection (the fourth pathway). Ecotropic MLV particles are internalized into intracellular vesicles, but the vesicles are not colocalized with clathrin [75]. Furthermore, the dynamin-dominant negative mutant does not inhibit ecotropic MLV infection in human HeLa cells expressing the ecotropic MLV receptor, suggesting that ecotropic MLV particles are internalized by clathrin- and dynamin-independent endocytosis [75]. In contrast, another report indicates that siRNA-mediated knockdown of dynamin and dynasore suppresses ecotropic MLV infection in mouse NIH3T3, rat XC, and human TE671 cells expressing the ecotropic receptor [52] (Table 3). As mentioned above, the internalization pathway of ecotropic MLV might be dependent on the cell lines used. ALV [76] and EIAV [77] infections occur through clathrin-dependent endocytosis. JSRV infection required dynamin-dependent endocytosis [69]. Taken together, these reports strongly support a notion that infections by many animal retroviruses occur through endosomes and require endosome acidification.

All of intracellular vesicles do not necessarily become acidic. For example, macropinosomes can be recycled to plasma membrane before their acidification, and recycling endosomes are formed from early endosomes and are transferred to plasma membrane [48]. Because many retroviral infections require endosome acidification, if viral particles are internalized into recycling endosomes, infectivity would decrease. To prevent this, the interaction between retrovirus

Table 3: Differential internalization pathways of HIV and MLV infections.

Viruses	Internalization pathway	Cell lines	Reference
Ecotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Dynamin-, clathrin independent	Human HeLa	[75]
Amphotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Caveolin dependent	Mouse NIH3T3	[74]
Polytropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
Xenotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
CD4-dependent HIV	Dynamin dependent	Human HeLa	[95]
	Clathrin dependent	Human primary T lymphocyte	[95–97]
	Dynamin-, Eps15 dependent	Human HeLa	[98]
	Dynamin-, Eps15 independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dynamin-, Eps15 dependent	Human 293T, HeLa, TE671	[21]

Env proteins and the infection receptors is speculated to induce a signal to trigger the acidification of virion-containing intracellular vesicles.

8. Cleavage of Retroviral Env Proteins by Cathepsins

Many retrovirus infections require endosome acidification. Influenza virus infection also requires endosome acidification, and treatment of influenza virus particles with low pH buffer activates its membrane fusion, indicating that low pH treatment directly induces conformational change of the influenza virus hemagglutinin to the fusion-active form. In contrast, low pH treatment of MLV particles does not activate the membrane fusion. Why does ecotropic MLV entry require endosome acidification?

There is another mystery of the endosome-mediated infection. Proteins internalized into acidic late endosomes/lysosomes are generally degraded by endosome proteases including cathepsins. The acidification inhibitors suppress the degradation in late endosomes/lysosomes [47]. If the retroviral particles are degraded in late endosomes/lysosomes, the acidification inhibitors would enhance retrovirus infection. However, the acidification inhibitors rather suppress the infection [52]. Therefore, it is suggested that the retroviral particles incorporated into late endosomes/lysosomes are not degraded. Why are the retroviral particles not degraded in acidic late endosomes/lysosomes?

The finding that endosomal cathepsin proteases are necessary for the ecotropic MLV infection [78, 79] like Ebola virus infection [80] has provided a clue to understanding the questions. Because cathepsin proteases are activated by acidification, the ecotropic MLV entry into host cytoplasm requires cathepsin activation by acidification. The weakly acidic condition (pH 6) in early endosomes cannot activate cathepsin proteinases [81], suggesting that ecotropic MLV infection occurs via late endosomes/lysosomes. The acidification inhibitors suppress MLV infections by attenuating cathepsin protease activation. The evidence that the acidification inhibitors do not suppress the ecotropic MLV infection in active cathepsin-containing medium further

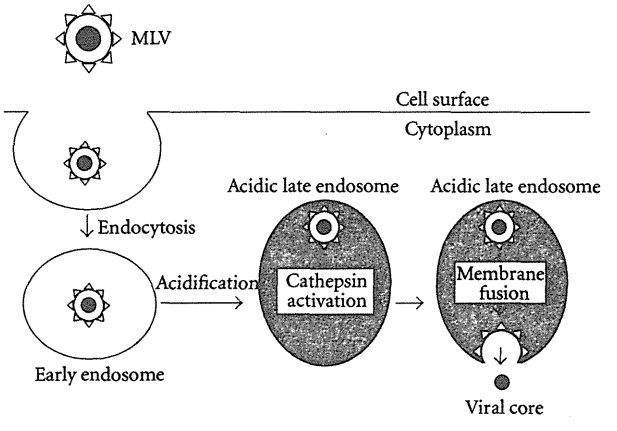

Figure 2: Entry pathway of ecotropic MLV in almost all susceptible cells. Blue area indicates acidic condition.

supports this conclusion [52]. Our current model for entry of ecotropic MLV is that cathepsin proteases digest MLV Env glycoproteins to generate fusion-active forms rather than to break them up completely, because treatment of ecotropic and amphotropic MLV particles with cathepsin B protease results in a few digested products of the Env proteins but not their disappearance [52, 79]. It is still unclear how the MLVs are not degraded in the late endosomes/lysosomes by other proteases.

In summary, the entry pathway of ecotropic MLV occurs as follows (Figure 2). Ecotropic MLV particles are internalized into endosomes, following the interaction of Env protein with the infection receptor. The viral particle-containing endosomes become acidic by V-ATPase. Cathepsin proteases are activated in the acidic late endosomes. The activated cathepsins cleave the ecotropic Env proteins to confer them fusion active. The cleaved Env proteins induce fusion between the viral envelope and host cell endosome membranes. Finally, the ecotropic MLV cores enter into host cytoplasm.

Although it is widely accepted that the ecotropic MLV infection requires endosome acidification and cathepsin proteases, the entry pathway of xenotropic MLV is not clear,

because of the contradictory reports [52, 53]. We have shown that xenotropic MLV infection requires endosome acidification and cathepsin proteases like the ecotropic MLV infection [52]. In sharp contrast, the Liu research group has reported that inhibitors of endosome acidification and cathepsin proteases do not inhibit the xenotropic MLV infection [53]. Different cell lines used in these studies may induce different entry pathways of the xenotropic MLV.

Unlike the ecotropic MLV entry, it has been reported that a low-pH pulse of JSRV particles overcomes the bafilomycin-mediated infection inhibition [69], EIAV infectivity is enhanced by low-pH treatment [67], and cell-cell fusion induced by the ALV Env protein is enhanced at low pH [55]. Additionally, analysis of the pH dependence of the foamy virus Env-mediated fusion in a cell-cell fusion assay revealed an induction of syncytium formation by a short exposure to acidic pH [66]. The low-pH treatment of these retroviruses may directly induce the conformational changes of their Env glycoproteins to fusion active forms without the proteolytic cleavage, like influenza virus.

9. PH-Independent MLV Infection in XC Cells

Although the acidification inhibitors attenuate the ecotropic MLV infection in almost all susceptible cells [49, 52], the inhibitors have no effect on the ecotropic MLV infection specifically in rat XC cells, suggesting that the ecotropic MLV infection in XC cells is independent of low pH [49] (Table 2). Furthermore, the R peptide-containing ecotropic Env protein can induce pH-independent syncytium formation in XC cells, but cannot in other susceptible cells [82, 83]. By these results, it had been widely thought that ecotropic MLV entry into XC cells occurs at cell surface membranes and does not require the internalization of virions into intracellular vesicles and acidification. This XC cell-specific pH-independent ecotropic MLV infection was one of the well-known mysteries in the MLV field [84, 85]. We found that a cathepsin inhibitor, CA-074Me, efficiently suppresses the ecotropic MLV infection in XC cells, like in other susceptible cells, suggesting that the ecotropic MLV infection in XC cells requires endosomal cathepsin proteases [52]. This result is inconsistent with the previous theory that the ecotropic MLV infection in XC cells does not occur through endosomes. Because the ecotropic MLV infection requires cathepsin proteases activated by endosome acidification, the acidification inhibitors would be proposed to suppress the MLV infection by attenuating cathepsin activation. However, the acidification inhibitors do not reduce cathepsin activity in XC cells, but do so in other cell lines, suggesting that cathepsin proteases are activated without endosome acidification in XC cells [52]. XC cells do not express so much cathepsin that activation is sufficient at suboptimal pH, because cathepsin activity of XC cells is comparable to that of NIH3T3 cells. These results prompted us to speculate that the ecotropic MLV infection in XC cells occurs through endosomes. The result that dynasore and siRNA-mediated knockdown of dynamin expression suppress the ecotropic MLV infection in XC cells strongly supports this hypothesis.

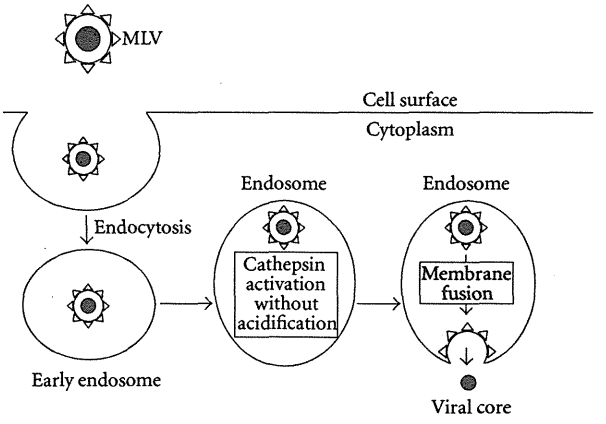

FIGURE 3: Entry pathway of ecotropic MLV in XC cells. Ecotropic MLV entry in XC cells may occur in acidic late endosomes, but endosome acidification is not required for the entry.

Taken together, the entry pathway of ecotropic MLV in XC cells is considered as follows (Figure 3). The ecotropic MLV particles are internalized into endosomes in XC cells, like in other susceptible cells. Cathepsin proteases are activated without endosome acidification. The activated cathepsins cleave the MLV Env protein, and the fusion between the viral envelope and host cell endosome membrane takes place for entry of the viral core into host cytoplasm. Because of the endosome acidification-independent activation of cathepsin proteases [52], the acidification inhibitors do not suppress the cathepsin protease activity and ecotropic MLV infection in XC cells. Additionally, this finding supports the above-mentioned hypothesis that the acidification inhibitors differentially affect retrovirus infections in different cell lines. The mechanism of acidification-independent cathepsin activation in XC cells is waiting to be resolved.

10. PH-Dependent Entry and PH-Independent Syncytium Formation by Retroviral Env Proteins

The R peptide-cleaved MLV Env protein induces the fusion between the viral envelope and host cell membranes for viral entry and syncytium formation in susceptible cells [33, 34]. Cells expressing the R peptide-truncated Env protein behave as large MLV particles and fuse with neighboring susceptible cells. Therefore, the syncytium formation by the retroviral Env proteins is thought to represent the membrane fusion in retroviral entry. Because the syncytium formation by the retroviral Env protein may contribute to the development of degenerative disorders like AIDS [28, 29], and because an endogenous retroviral Env protein (syncytin) induces syncytiotrophoblast formation [86], the elucidation of mechanism of retroviral Env-induced syncytium formation is essential to understand retroviral pathogenesis and placenta development. The MLV entry into host cells is dependent on low pH, but the syncytium formation by the R peptide-truncated Env protein is independent [33].