HIV-1 Vpr and p21 restrict LINE-1 mobility

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ABSTRACT

Long interspersed element-1 (LINE-1, L1) composes 17% of the human genome. However, genetic interactions between L1 and human immunodeficiency virus type 1 (HIV-1) remain poorly understood. In this study, we found that HIV-1 suppresses L1 retrotransposition. Notably, HIV-1 Vpr strongly inhibited retrotransposition without inhibiting L1 promoter activity. Since Vpr is known to regulate host cell cycle, we examined the possibility whether Vpr suppresses L1 retrotransposition in a cell cycle dependent manner. We showed that the inhibitory effect of a mutant Vpr (H71R), which is unable to arrest the cell cycle, was significantly relieved compared with that of wild-type Vpr, suggesting that Vpr suppresses L1 mobility in a cell cycle dependent manner. Furthermore, a host cell cycle regulator p21WAF1 strongly suppressed L1 retrotransposition. The N-terminal kinase inhibitory domain (KID) of p21 was required for this inhibitory effect. Another KID-containing host cell cycle regulator p27KIP1 also strongly suppressed L1 retrotransposition. We showed that Vpr and p21 coimmunoprecipitated with L1 ORF2p and they suppressed the L1 reverse transcriptase activity in LEAP assay, suggesting that Vpr and p21 inhibit ORF2p-mediated reverse transcription. Altogether, our results suggest that viral and host cell cycle regulatory machinery limit L1 mobility in cultured cells.

INTRODUCTION

Long interspersed element-1 (LINE-1, L1) is an active and autonomous non-long terminal repeat (LTR) retrotransposon composing 17% of the human genome (1–3). L1 encodes two open reading frames (ORFs), ORF1p with RNA binding domain and nucleic acid chaperone activity, and ORF2p with endonuclease and reverse transcriptase activities required for its retrotransposition (1,2,4,5). L1 transcription occurs through promoter activity located in its 5′UTR (6). Several transcription factors including p53 (7), RUNX3 (8), SOX11 (9) and YY1 (10,11) positively regulate the L1 transcription (12). On the other hand, SRY (9) and SOX2 (13) negatively regulate the L1 transcription. L1 RNA assembles with ORF1p and ORF2p to form a ribonucleoprotein (RNP) complex in the cytoplasm (14). Then, L1-RNP complex enters the nucleus in which genomic integration occurs by a mechanism termed target-primed reverse transcription (TPRT). During TPRT, the L1 endonuclease creates a nicked DNA that serves as a primer for reverse transcription of L1 RNA, leading to integration of L1 cDNA into the human genome (15). Although L1 expression and retrotransposition can occur during early embryogenesis (16–18) and gametogenesis (18,19), L1 transcription is largely repressed by DNA methylation in somatic cells (19,20). In addition to the epigenetic control of L1 expression, L1 retrotransposition is controlled by several host restriction factors such as APOBEC3G (A3G), APOBEC3F (A3F) and MOV10 (12,21–27). A3G was first identified as anti-human immunodeficiency virus type 1 (HIV-1) restriction factor (28) and HIV-1 restriction requires A3G cytidine deaminase activity (29,30). A3G restricts exogenous retroviruses, hepatitis B virus (HBV), and endogenous retroelements, such as L1, Alu, SVA and HERVs (21,29,31–34). However, the A3G cytidine deaminase activity is dispensable for L1 restriction. Escape of these control pathways can lead to de novo L1 retrotransposition in somatic cells that could contribute to mutagenesis and genomic instability leading to cancer (35–38). L1 retrotransposition can also generate mutations of genes in the germ line or during develop.
opment that could contribute to diseases (39, 40). Therefore, L1 must be regulated during normal development.

HIV-1 is a retrovirus, which encodes three structural proteins, group-specific antigen (Gag), polymerase (Pol), and envelope (Env), two regulatory proteins, Tat and Rev, and four accessory proteins, Vif, Vpu, Vpr and Nef. The gene expression of HIV-1 is transcriptionally regulated by Tat through its binding to a nascent HIV-1 trans-activation responsive (TAR) RNA (41, 42), and post-transcriptionally by Rev through its interaction with Rev-responsive element (RRE) RNA in the env gene (43–45). Rev forms a complex with CRM1–Ran–GTP and enhances the nuclear export of HIV-1 mRNA (43–45). In addition, several host DEAD-box RNA helicases cooperate to modulate HIV-1 Rev function (46–50). HIV-1 Vpr is a virion-associated nuclear protein with multiple functions (51, 52). Vpr facilitates HIV-1 infection of nondividing cells by regulating the nuclear import of the HIV-1 pre-integration complex (PIC). Vpr also induces cell cycle arrest at the G2 phase in proliferating infected cells and stimulates the LTR-directed gene expression (53). Following HIV-1 entry, its own reverse transcriptase synthesizes a DNA copy of the HIV-1 genomic RNA. Integration of a DNA copy of the viral RNA genome is a crucial step in the life cycle of HIV-1. Therefore, both HIV-1 and L1 might mutually influence their mobility. However, interactions between HIV-1 and L1 are not well understood. Therefore, we investigated a cross talk of HIV-1 with L1 in this study.

MATERIALS AND METHODS

Cell culture

293T, TET293T, P4.2 and TZM-bl cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA, USA) with high glucose (4.5 g/ml penicillin/streptomycin. Details of specific transfection conditions for each experiment are provided in the figure legends.

Plasmid construction

To construct pcDNA3-HA-ORF1 or pcDNA3-ORF1-HA, a DNA fragment encoding ORF1p was amplified from pEGFP-1LP wt (54) by PCR using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and the following pairs of primers: ORF1-HA, 5′-CCGCGCCCGCTACGATGTCGGG-3′ (Forward), 5′-CCGCGCCCGCTACGATGTCGGGACAGCAG-3′ (Reverse); ORF1-HA, 5′-GGGAAAAAACAGAACAAGCCCCAGAAG-3′ (Forward), 5′-GGGAAAAAACAGAACAAGCCCCAGAAGAC-3′ (Reverse); EGFP-Vpr, 5′-CGTCGAGAAGATGGAAAGCCCCAGAAG-3′ (Forward), 5′-CGTCGAGAAGATGGAAAGCCCCAGAAGAC-3′ (Reverse). The obtained DNA fragments were subcloned into the BamHI–XbaI sites of the pcDNA3-HA vector or the XhoI–BamHI sites of the pcDNA3-HA vector. To construct pAIP-Vpr WT or pcDNA3-ORF1-HA by PCR using KOD-Plus DNA polymerase and the following pair of primers: 5′-CGTCGAGAAGATGGAAAGCCCCAGAAG-3′ (Forward), 5′-CGTCGAGAAGATGGAAAGCCCCAGAAGAC-3′ (Reverse). The obtained DNA fragments were subcloned into the BamHI–XbaI sites of the pcDNA3-HA vector. To construct pAIP-Vpr WT or pcDNA3-ORF1-HA by PCR using KOD-Plus DNA polymerase and the following pairs of primers: HA-Vpr WT, 5′-CCGCCCGCGCCGCAAGATGGAAAG-3′ (Forward), 5′-CCGCCCGCGCCGCAAGATGGAAAGAC-3′ (Reverse); HA-Vpr H71R, or pEGFP-Vpr, a DNA fragment encoding Vpr WT or H71R was amplified from pcDNA3-HA-Vpr WT or pcDNA3-HA-Vpr H71R by PCR using KOD-Plus DNA polymerase and the following pair of primers: 5′-CCGCCCGCGCCGCAAGATGGAAAG-3′ (Forward), 5′-CCGCCCGCGCCGCAAGATGGAAAGAC-3′ (Reverse). The obtained DNA fragments were subcloned into the BamHI–XbaI sites of the pcDNA3-HA vector or the XhoI–BamHI sites of the pcDNA3-HA vector.

RNA interference

Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to p21 in a lentiviral vector: 5′-GATCCCCGACCATGTGGAGACTGTCAAGATGACGAGGCCGGGATGAGT-3′ (sense), 5′-AGCTTTTCCAAAAAGACCAATGTTATGACGAGGCCGGGATGAGT-3′ (antisense). The oligonucleotides above were annealed and subcloned into the BglII–HindIII sites, downstream from an RNA polymerase III promoter of pSUPER (57), to generate pSUPER-p21. To construct pLenti-p21, the BamHI–SalI sites of the pSUPER-p21 were subcloned into the BamHI–SalI sites of the pcDNA3-HA vector.

Lentiviral vector production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously.
The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMVΔR8.74 (59, 60) and the VSV-G-envelope-expressing plasmid pMD2G as well as lentiviral vector pLV-p21i or pAIP (55) into 293T cells with FuGENE6 (Promega, Madison, WI, USA) or TransIT-LT1 (Mirus Bio LLC, Madison, WI 53711, USA) transfection reagents.

**HIV-1 infection**

Different full-length HIV-1 molecular clones used for transfection were as follows: pR9 (59, 60), pNL4-3 (61), pJR-FL (62) and pAD8 (63). Recombinant HIV-1 was prepared as construct pCMV transient transfection of the second-generation packaging expressing plasmid pMD2G as well as lentiviral vector infections were as follows: pR9 (59, 60), pNL4-3 (61), pJR-FL or pAD8. Culture media were replaced with fresh media 24 h after transfection, and the cells were cultured for an additional 48 h. Then, the supernatants containing recombinant viruses were clarified by centrifugation and filtration with 0.45 μm filter (Kurabo, Osaka, Japan), and stored at −80°C before use. Released HIV-1 virions were collected by centrifugation of the culture supernatants from transfected 293T cells at 20,000 × g for 2 h at 4°C. The pellets containing virions were dissolved in the lysis buffer and then subjected to western blot. HIV-1 infection was performed as described previously (64). P4.2, CD4+ HeLa cells were incubated with the supernatants of 293T cells containing HIV-1 at the indicated multiplicity of infection (MOI), and the cells were cultured for an additional 48 h.

**Western blot analysis**

Cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet (N) P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HIV-1 p24 (65-005; Bioa- sciences) as secondary antibodies.

**Luciferase assay**

293T cells were seeded in 24-well plates at 2 × 10^4 cells per plate and then transfected the next day with plasmids using FuGENE6 (Promega, Madison, WI, USA) or TransIT-LT1 transfection reagent. Luciferase assays were performed 72 h after transfection using luciferase assay reagent according to the manufacturer’s instructions (Promega). All transfections were performed using equal number of tested plasmid DNA molecules, with the addition of empty vector into the transfection mixtures to compensate for plasmid size differences and reach equal amounts of DNA quantities per condition. Results were obtained through three independent transfections. A Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to measure luciferase activities.

**Cell cycle analysis**

For cell cycle analysis, cells were fixed in 70% ethanol at 4°C overnight and stained with propidium iodide (PI, 10 μg/ml) (66). The DNA content in each cell was analyzed on LSRII flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed on Flowjo software (Tree Star, San Carlos, CA).

**Immunoprecipitation**

Cells were lysed in buffer containing 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10 mM NaF, 1 mM DTT and 1 mM PMSF. Lysates were pre-cleared with 30 μl of protein-G-Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden). Pre-cleared supernatants were incubated with 5 μg of either anti-HA antibody (3F10; Roche Diagnostics, Mannheim, Germany) or anti-FLAG antibody (M2, Sigma) at 4°C for 1 h. Following absorption of the precipitates on 30 μl of protein-G-Sepharose resin for 1 h, the resin was washed four times with 700 μl of lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2× Laemml sample buffer. The proteins were then subjected to SDS-PAGE, followed by immunoblot analysis.

**Immunofluorescence and confocal microscopy analysis**

293T cells were grown on Lab-Tek 2 well chamber slide (Nunc, Thermo) at 2 × 10^4 cells per well and transfected the next days using TransIT-LT1 transfection reagent. Two days after transfection, the cells were fixed in 3.6% formaldehyde in 1× phosphate-buffered saline (PBS), permeabilized in 0.1% NP-40 in 1× PBS at room temperature, and incubated with anti-HA antibody (HA-7; Sigma) at 4°C for 1 h. Following absorption of the precipitates on 30 μl of protein-G-Sepharose resin for 1 h, the resin was washed four times with 700 μl of lysis buffer. Proteins were eluted by boiling the resin for 5 min in 2× Laemml sample buffer. The proteins were then subjected to SDS-PAGE, followed by immunoblot analysis.

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Real-time RT-PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen) and treated with Turbo DNA-free kit (Life Technology). The total RNA was reverse transcribed with oligo(dT)12-18 primer (Life Technology). The quantitative RT-PCR analysis for LINE-1 mRNA was performed by real-time LightCycler PCR (Roche). We used the following forward and reverse primer sets:

5′UTR L1 Hs, GGGAGGAGAGCCCAAGATG (Forward), ACACGGCCTGCCGCCCCACTG (Reverse);

ORF1 L1 Hs, AAAACGCAGACGGCCTC (Forward), GTGGCCGCGCTGTTCCGGTAG (Reverse);

ORF1 L1PA, GAACGCCACAAAGATACTCC (Forward), GTTTGAATGTCCTCCCGTAG (Reverse);

ORF1 L1 Hs, AAAACGCAGACGGCCTC (Forward), CTCTTCTGGCCTGTAGGGTTTCTG (Reverse);

β-actin, TGACCCCACCCCACTCTG (forward), AAGCTGTAAGCAGCGTCTCGG (reverse).

Purification of L1 RNPs

L1 RNPs were prepared as previously described with minor modifications (65,67). Briefly, 293T cells were seeded in 10 cm plates at 3 × 10⁶ cells per plate and then transfected the next days with 24 μg of plasmid using the calcium phosphate-mediated method (68). Culture medium was changed 5h later. Cells were collected 5 days post-transfection by trypsinization and were then lysed in CHAPS extract buffer containing 10 mM Tris–HCl pH 7.5, 0.5% CHAPS (w/v), 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, protease inhibitors cocktail (Roche) and 1 mM DTT. After incubation at 4°C for 15 min, cell debris was removed by centrifugation at 20,000 × g for 15 min. A sucrose cushion was prepared with 8.5% and 17% (w/v) sucrose cushion buffer containing 10 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 1 mM EGTA, protease inhibitors cocktail (Roche) and 1 mM DTT. For each condition, 1 ml of cell lysate was loaded on a sucrose cushion. Cellular RNPs were pelleted by ultracentrifugation at 178,000 g for 4°C for 2 h. The pellets were resuspended in 50 μl of H₂O. Quantification was performed using the Bradford assay (Bio-rad). RNP fractions were diluted at 1 μg/μl in H₂O and aliquoted before use or storage at −80°C.

L1 element amplification protocol (LEAP) assay

LEAP assay was performed as previously described (65,67). Briefly, L1 reverse transcription was carried out at 37°C for 1 h in 50 μl reaction mixture containing 0.75 μg of RNPs, 50 mM Tris–HCl pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.05% (v/v) Tween-20, 10 mM DTT, 20 U R Nasin (Promega), 0.2 mM dNTPs and 0.4 μM RACE primer (5′-GCGAGCACAGAATTATACGACT CACTATAGGTATTATTTTTTVN-3′).

1 μl of the LEAP reaction were PCR-amplified in 50 μl reactions containing 0.2 mM dNTPs, 3 mM MgCl₂, 10 pmol of primers LOU312 (5′-GGGAGGGTTATCATAAGGATACGACT-3′), LOU851 (5′-GGGGTGAAATCGATAAGGATGCTGACGAC-3′) and 2 U of Platinum Taq Polymerase (Life technologies). A first step at 94°C for 2 min was followed by 35 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 30 s and by a final step at 72°C for 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis in 0.5× TBE buffer.

RNA extraction and RT-PCR

Total RNA was extracted from 15 μg of L1 RNP fraction using TRI reagent (Molecular Research Center Inc). DNase treatment was performed at 37°C for 30 min in a 10 μl reaction mixture containing 1 μg of extracted RNA, 1 U of RQ RNase-free DNase (Promega), 40 mM Tris–HCl pH 8.0, 10 mM MgSO₄ and 1 mM CaCl₂. Then, cDNA synthesis was performed at 50°C for 1 h in 20 μl reaction mixture containing 6 μl of DNase-treated RNA, 50 pmol of RACE primer, 10 mM dNTPs, 40 U R NaseOUT recombinant ribonuclease inhibitor (Life Technologies), 200 U of SuperScript III Reverse Transcriptase (Life technologies), 50 mM Tris–HCl pH8.0, 75 mM KCl, 3 mM MgCl₂ and 5 mM DTT. After the first-strand cDNA synthesis, PCR amplification was performed as described above but with only 30 cycles of amplification and using primers LOU312 and LOU851 for L1, and LOU1071 (5′-GGTTTCTCAGGATTGTGTTGTA GATC-3′) and LOU1072 (5′-GAAGATGGTGATGGGATTCC-3′) for GAPDH.

Retrotransposition assays

LINE-1 retrotransposition assays consist of the expression in cells of a retrotransposition-competent L1 element equipped with an indicator cassette for which the reporter gene is expressed only after a cycle of retrotransposition (54,70–72). The indicator cassette consists of a reporter gene (e.g. EGFP, firefly luciferase) with its own promoter and polyadenylation signal. It is introduced in the 3′UTR of an active L1, in the opposite transcriptional orientation compared to L1 (Figure 1A and B). The reporter gene is interrupted by an intron with splice donor and splice acceptor sites in the transcriptional orientation of the L1. Therefore, the only way to express the reporter gene is when L1 RNA is expressed from its promoter, splicing occurs to remove the intron, and then retrotransposition takes place, leading to the insertion at a genomic locus of the reporter gene. In absence of the original intron, the reporter gene can now be expressed from the integration site.

The EGFP-based retrotransposition assay was performed as followed: 293T cells were seeded in 12-well plates at 5 × 10⁴ cells per plate and transfected the next day using FuGENE6 with 500 ng of either an EGFP-based retrotransposition reporter pL1RP(JM111)-EGFP (54) or its corresponding negative control pL1RP(JM111)-EGFP which contains two missense mutations (ARR at residues 260–262) in the ORF1 coding region known to abolish retrotransposition (54). Three days after transfection, retrotransposition efficiency was determined by GFP fluorescence-activated cell sorter (FACS) analysis with Guava easyCyte flow cytometer (Merck Millipore, Billerica, MA, USA).

The luciferase-based retrotransposition assay was performed using the firefly luciferase-based retrotransposition reporter pYX014 plasmid (72). pYX014 is a dual-luciferase
Figure 1. Suppression of L1 retrotransposition by HIV-1. (A) Schematic representation of the EGFP-based retrotransposition reporter cassette, pL1RP-EGFP (54). The EGFP reporter is introduced in the 3′UTR of L1, in opposite orientation. It is also interrupted by an intron with splice donor (SD) and splice acceptor (SA) in the transcriptional orientation of the L1. 293T cells (5 × 10⁴ cells/well) were co-transfected with HIV-1 molecular clone (R9, NL4-3 or JR-FL) (500 ng) and either pL1RP-EGFP or its mutant pL1RP(JM111)-EGFP lacking the ability of retrotransposition (500 ng). Three days after transfection, retrotransposition efficiency was determined by GFP fluorescence-activated cell sorter (FACS) analysis with Guava easyCyte flow cytometer (Merk Millipore, Billerica, MA, USA). Experiments were done in triplicate and graph shows the mean (±SEM) percentage of GFP positive cells (*P < 0.05). (B) Schematic representation of the firefly luciferase-based retrotransposition reporter cassette, pYX014 (72). The pYX014 plasmid is a dual-luciferase reporter, in which firefly luciferase is used as the retrotransposition indicator and Renilla luciferase is encoded on the same plasmid backbone for normalization. 293T cells were seeded in 24-well plates at 2 × 10⁴ cells per plate and then transfected the next day with pYX014 (100 ng) using TransIT-LT1 transfection reagent. Dual luciferase assays were performed 72 h after transfection using luciferase assay reagent according to the manufacturer’s instructions (Promega). A Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to measure both firefly and Renilla luciferase activities. Firefly luciferase activity was normalized with Renilla luciferase activity.

RESULTS

HIV-1 Vpr suppresses L1 retrotransposition

To investigate the potential role of HIV-1 expression in L1 retrotransposition, three different HIV-1 molecular clones (R9, NL4-3 and JR-FL, respectively) (59–62) were co-transfected in 293T cells with pL1RP-EGFP plasmid (Figure 1A), which contains an EGFP-based retrotransposition reporter cassette in the antisense orientation (54). The presence of an intron in the opposite transcriptional orientation of the reporter prevents EGFP expression from the plasmid. GFP positive cells can only arise after expression, splicing and retrotransposition of the labeled L1RP copy into new genomic location. The use of a retrotransposition-deficient L1 expressing plasmid, pL1RP(JM111)-EGFP, serves as negative control of the assay (54). We analyzed by flow cytometry the level of GFP expression and showed that reporter in which firefly luciferase is used as the retrotransposition indicator and Renilla luciferase is encoded on the same plasmid backbone for normalization. 293T cells were seeded in 24-well plates at 2 × 10⁴ cells per plate and then transfected the next day with pYX014 (100 ng) using TransIT-LT1 transfection reagent. Dual luciferase assays were performed 72 h after transfection using luciferase assay reagent according to the manufacturer’s instructions (Promega). A Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to measure both firefly and Renilla luciferase activities. Firefly luciferase activity was normalized with Renilla luciferase activity.

Statistical analysis

The statistical significance of the inter-sample differences was determined using the paired Student’s t test. P values of <0.05 were considered statistically significant.
all HIV-1 clones could strongly suppress the retrotransposition of L1 (Figure 1A). To confirm these findings, we also assayed retrotransposition using the pYX014 plasmid, which has a firefly luciferase-based retrotransposition reporter cassette (72). The pYX014 plasmid is a dual-luciferase reporter in which firefly luciferase is used as the retrotransposition indicator and Renilla luciferase is encoded on the same plasmid backbone for normalization (Figure 1B). Similar to the EGFP reporter, the firefly luciferase activity can only be detected once L1 retrotransposition occurred. Again, all HIV-1 clones could strongly suppress the luciferase-based L1 retrotransposition activity (Figure 1B). We next examined whether HIV-1 infection affects L1 retrotransposition activity. CD4+ HeLa P4.2 cells were infected with HIV-1 (R9 or NL4-3) at the indicated multiplicity of infection (MOI) (Figure 1C and D). The next day, the luciferase-based retrotransposition reporter plasmid pYX014 was transfected into the HIV-1-infected cells. Two days post-transfection, we measured luciferase activities and observed a three-fold reduction of L1 retrotransposition at the higher MOI for both R9 and NL4-3, indicating that HIV-1 infection also modulates L1 mobility (Figure 1C and D). Since we did not infect equalized viral input (p24 amount) of HIV-1 between R9 and NL4-3 strains, the suppression of L1 retrotransposition seems to vary based on the MOI (Figure 1C and D). Subsequently, we used individual HIV-1 protein-expressing plasmids to determine which of the following HIV-1 protein suppresses L1 retrotransposition: Vpr, Tat, Rev, integrase (IN), Env and Nef. Using the EGFP-based retrotransposition reporter, we showed that only Vpr could strongly suppress L1 retrotransposition (Figure 2A). We also noticed that Tat weakly suppress L1 retrotransposition (Figure 2A). Moreover, Vpr suppressed L1 retrotransposition activity in a dose-dependent manner (Figure 2B). Likewise, Vpr could strongly suppress the luciferase-based L1 retrotransposition activity (Figure 2C). To test if Vpr is affecting the L1 promoter activity, we used pYX017 plasmid in which the L1RP 5′ UTR (present in pYX014) is replaced by a strong CAG promoter resulting in an increased retrotransposition activity (72) (Figure 2D). Despite the change of promoter, Vpr can also suppress L1 retrotransposition when expressed from pYX017 (Figure 2D), suggesting that Vpr does not affect the endogenous L1 promoter activity. Since HIV-1 Vpr is known to regulate the host cell cycle by arresting cells at G2 phase (53), we examined whether Vpr suppresses L1 retrotransposition in a cell cycle dependent manner. To this end, we used a mutant Vpr (H71R) unable to arrest cell cycle (53). Consistent with the previous report, we confirmed that wild-type Vpr (WT) arrested cell cycle at G2 (Figure 3A and B), while the H71R mutant failed to arrest cell cycle (Figure 3C). As a control, we observed similar levels of protein expression for both Vpr (WT) and the mutant Vpr (H71R) in transfected cells (Figure 3D). Although Vpr WT suppresses L1 retrotransposition activities from both pYX014 and pYX017, the inhibitory effect of Vpr (H71R) was significantly relieved compared with that of Vpr (WT) (Figure 3E and F), suggesting that Vpr suppresses L1 mobility in a cell cycle dependent manner.

Figure 2. Suppression of LINE-1 retrotransposition by HIV-1 Vpr. (A) 293T cells (5 × 10^4 cells/well) were co-transfected with each HIV-1 protein-expressing plasmid, Vpr, Tat, Rev, IN, Env and Nef (500 ng), and either pL1RP-EGFP or pL1RP(JM111)-EGFP (500 ng). Three days after transfection, retrotransposition efficiency was determined by GFP FACS analysis. Experiments were done in triplicate and graph shows the mean (±SEM) percentage of GFP positive cells (* P < 0.05). (B) Suppression of LINE-1 retrotransposition by HIV-1 Vpr in a dose-dependent manner. 293T cells (5 × 10^4 cells/well) were co-transfected with the indicated amounts (ng) of pCMX-Vpr96 and either pL1RP-EGFP or pL1RP(JM111)-EGFP (500 ng). Relative retrotransposition efficiency (fold) among three replicates (±SEM) is shown (* P < 0.05). (C, D) 293T cells (2 × 10^4 cells/well) were co-transfected with pCMX-Vpr96 (100 ng) and either pYX014 (C) or pYX017 (100 ng) (D) in triplicate. Three days after transfection, luciferase assays were performed. Graph shows the mean (±SEM) firefly luciferase activity normalized with Renilla luciferase activity with the condition without Vpr set to 100% (* P < 0.05).

p21Waf1 and p27Kip1, host cell cycle regulators, suppress L1 retrotransposition

Since HIV-1 Vpr restricted L1 retrotransposition in a cell cycle dependent manner (Figure 3), we examined whether host cell cycle regulators, such as p21Waf1/Cip1 (p21) and p27

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Kip1 (p27) (73–77) might suppress L1 mobility. To test this possibility, several plasmids expressing tumor suppressor-related proteins were independently co-transfected with pL1RP-EGFP plasmid into 293T cells. GFP fluorescence was then analyzed by flow cytometry to monitor the impact on L1 retrotransposition. Notably, p21 strongly suppressed L1 retrotransposition (Figure 4A). p53 and p73, which transcriptionally induce p21, also significantly suppressed L1 retrotransposition (Figure 4A). p21 has an N-terminal kinase inhibitory domain (KID) and a C-terminal PCNA-binding domain (Figure 4B) (56). Therefore, we used the N-terminal half (p21N) or the C-terminal half of p21 (p21C) to determine which domain is important for an inhibitory effect on L1 retrotransposition (Figure 4B). Our results showed that p21N but not p21C strongly suppressed L1 retrotransposition indicating that the kinase inhibitory domain is important for the inhibitory effect (Figure 4C and D). In this regard, p21N is known to inhibit cell growth whereas p21C does not (56). Consistent with this result, p27 harboring the kinase inhibitory domain also strongly suppressed L1 retrotransposition (Figure 4C and D). To further confirm this observation, we established a p21 knockdown 293T cell line using lentiviral vector stably expressing shRNA targeted to p21 (p21KD in Figure 4E). As expected, the L1 retrotransposition efficiency was elevated in p21 knockdown 293T cells (Figure 4F and G), even though we observed no significant effect of p21 knockdown on the 293T cell growth. Thus, host cell cycle inhibitors p21 and p27 restrict L1 mobility.

Incorporation of L1 ORF1p into HIV-1 virions

Since it has been indicated that several P-body and stress granule components, such as APOBEC3G, APOBEC3F and MOV10, are incorporated into HIV-1 virions and affect HIV-1 infectivity or production (78–82), we examined whether ORF1p is incorporated into HIV-1 virions and modifies the HIV-1 infectivity. Notably, we noticed that HA-tagged ORF1p (either in N- or C-terminus) are incorporated into the HIV-1 virions (NL4-3 and JR-FL), respectively (Figure 5A). We also found that the incorporation of ORF1p into HIV-1 virions was independent of HIV-1 accessory proteins including Nef, Vpr, Vpu and Vif, because the mutant viruses lacking the expression of each viral gene still retained the ability to incorporate ORF1p (Figure 5B). However, we failed to detect ORF2p and L1 RNA in HIV-1 virion (data not shown). On the other hand, ORF1p affected neither the intracellular nor extracellular HIV-1 p24 protein levels, suggesting that ORF1p does not negatively impact HIV-1 replication (Figure 5A). Furthermore, the incorporated ORF1p-HA did not drastically affect the HIV-1 infectivity, as measured by luciferase assay with two independent infectious HIV-1 molecular clones (NL4-3 and JR-FL) (61, 62) (Figure 5C and D). However, we noticed a modest suppressive effect of HA-ORF1 on the infectivity of NL4-3 but not JR-FL. Since ORF1p is an RNA-binding and nucleic acid chaperone protein, we also examined whether ORF1p modulates the HIV-1 Tat-mediated HIV-1 transcription using the HIV-1 LTR Luciferase reporter assay (83–85) or HIV-1 Rev-dependent nuclear ex-
Figure 4. p21 and p27 suppress L1 retrotransposition. (A) Potential effect of tumor suppressor proteins on L1 retrotransposition. 293T cells (5 × 10^4 cells/well) were co-transfected with each plasmid expressing tumor suppressor-related protein, p53 (115), p73α (116,117), p73β (116,117), p21 (56,118), Chk2 (119,120), Rb, ATM (121,122), or ATR (500 ng), and either pL1rp-EGFP or pL1rp(JM111)-EGFP (500 ng). Three days after transfection, retrotransposition efficiency was determined by GFP FACS analysis. Experiments were done in triplicate and graph shows the mean (±SEM) percentage of GFP positive cells (*P < 0.05). (B) Schematic representation of p21, p21 mutants (p21N and p21C), and p27. KID: kinase inhibitory domain, PCNA: PCNA-binding domain, QT: glutaminethreonine-rich domain. Protein expression levels of p21, p21N, p21C, and p27. 293T cells (2 × 10^5 cells/well) were transfected with pcDNA3, pcDNA3p21 (56), pcDNA3p21N (56), pcDNA3p21C or pcCMXp27. Western blotting of the cell lysates with anti-p21, anti-27, or anti-β-actin antibodies is shown. (C, D) KID is required for inhibitory effect on L1 retrotransposition. 293T cells (2 × 10^5 cells/well) were co-transfected with pcDNA3p21, pcDNA3p21N, pcDNA3p21C or pcCMXp27 (100 ng) and either pYX014 (C) or pYX017 (D) (100 ng) in triplicate. Three days after transfection, luciferase assays were performed. Graph shows the mean (±SEM) relative luciferase activity normalized with Renilla luciferase activity in Relative Luminometer Units (*P < 0.05). (E) Inhibition of p21 expression by shRNA-producing lentiviral vector. Western blotting of cell lysates with anti-p21 or anti-β-actin antibodies is shown. (F, G) Enhancement of L1 retrotransposition efficiency in p21 knockdown cells. Control (ctrl) or p21 knockdown (p21KD) 293T cells (2 × 10^5 cells/well) were transfected with either pYX014 (F) or pYX017 (G) (100 ng) in triplicate. 24h after transfection, luciferase assays were performed, and results were plotted as described above (*P < 0.05).
Figure 5. Incorporation of L1 ORF1p into HIV-1 virions. (A) 293T cells (2 × 10^5 cells/well) were co-transfected with 2 μg of pcDNA3-HA-ORF1 or pcDNA3-ORF1-HA and 2 μg of pNL4-3 or pJR-FL. Western blotting of cell lysates or virion in the concentrated culture supernatants with anti-HA, anti-HIV-1 p24, or anti-β-actin antibodies is shown. (B) HIV-1 accessory protein-independent incorporation of ORF1p into HIV-1 virions. 293T cells (2 × 10^5 cells/well) were co-transfected with 2 μg of HIV-1 AD8 wild-type molecular clone (WT) (63) or HIV-1 mutants (ΔVif, ΔVpr, ΔVpu, or ΔNef) and 2 μg of pcDNA3-ORF1-HA. HIV-1 virions were then produced in the culture supernatants. Western blotting of HIV-1 in the concentrated culture supernatants with either anti-HA or anti-HIV-1 p24 antibody is shown. (C, D) Incorporation of ORF1p does not affect the HIV-1 infectivity. TZM-bl cells (1 × 10^5 cells/well) were inoculated with 100 μl of the culture supernatants of 293T cells co-transfected with pNL4-3 (C) or pJR-FL (D), and pcDNA3-HA-ORF1 or pcDNA3-ORF1-HA as shown in (A). 24 h post-infection, luciferase assays were performed. Graph shows the mean (±SEM) firefly luciferase activity in Relative Luminometer Units (*P < 0.05). (E) L1 ORF1p does not affect the Tat-mediated HIV-1 transcription. 293T cells (2 × 10^4 cells/well) were co-transfected with HIV-1 LTR-Luc (100 ng), pcDNA3-HA-ORF1 (100 ng), pcDNA3-ORF1-HA, and/or pcDNA3-Tat101-FLAG (123) (100 ng) in triplicate. 24h after transfection, luciferase assays were performed, and results were plotted as described above (*P < 0.05). (F) L1 ORF1p does not affect the Rev-mediated nuclear export function. 293T cells (2 × 10^4 cells/well) were co-transfected with pDM628 (47) (100 ng), pcDNA3-HA-ORF1 (100 ng), pcDNA3-ORF1-HA, and/or pcRev (47) (100 ng) in triplicate. 24 h after transfection, luciferase assays were performed, and results were plotted as described above (*P < 0.05).

port function using the Rev-dependent luciferase-based reporter plasmid pDM628 (47,49). Our results showed that ORF1p did not affect either Tat or Rev function (Figure 5E and F).

Vpr and p21 do not suppress L1 transcription and ORF1p expression

To determine whether Vpr and p21 suppress L1 mobility by inhibiting the L1 promoter activity, we examined L1 mRNA expression by real-time RT-PCR. Both p21 and Vpr did not affect the level of L1 mRNA from the L1 5'UTR promoter (pYX014) (Figure 6A). HIV-1 clones (R9 and NL4-3) enhanced L1 transcription two days post-transfection (Figure 6A). Similarly, Vpr did not suppress the level of endogenous L1 mRNA (Figure 6B). Thus, Vpr and p21 may suppress L1 mobility post-transcriptionally. Since Vpr is known to enhance p21 promoter activity (86,87), we wished to confirm this regulation in 293T cells. Indeed, we observed that
Figure 6. Vpr and p21 do not suppress L1 transcription. (A) The level of L1 ORF1 mRNA in 293T cells co-transfected with pYX014 and pcDNA3p21, pCMX-Vpr96, pR9, or pNL4-3 was monitored by real-time LightCycler PCR two days post-transfection, respectively. Experiments were done in triplicate, and graph shows the mean percentages (±SEM) of L1 ORF1 mRNA normalized with β-actin mRNA, with the pYX014 condition set at 100% (*P < 0.05).

(B) The level of endogenous L1 mRNA in 293T cells transfected with pCMX-Vpr96 was monitored by real-time LightCycler PCR. Primer pairs targeting primate (L1PA - ORF1) or human (L1Hs - ORF1 and 5′ UTR) L1 subfamilies were used. Experiments were done in triplicate, and graph shows the mean percentages (±SEM) of L1 mRNA in presence of Vpr normalized with both β-actin mRNA and the ‘no Vpr’ condition for each primer pair (*P < 0.05).

(C) Effect of Vpr on p21 promoter activity. 293T cells (2×10^4 cells/well) were co-transfected with pWAF1-Luc (100 ng) and pCMX-Vpr96 (100 ng) in triplicate. 24h after transfection, luciferase assays were performed. Graph shows the mean (±SEM) firefly luciferase activity in Relative Luminometer Units (*P < 0.05).

(D), (E) Vpr suppresses L1 retrotransposition efficiency in a p21-independent manner. p21 (p21KD) knockdown 293T cells (2×10^4 cells/well) were co-transfected with pCMX-Vpr96 (100 ng) and either pYX014 (D) or pYX017 (E) (100 ng) in triplicate. 24h after transfection, luciferase assays were performed. Graph shows the mean (±SEM) firefly luciferase activity normalized with Renilla luciferase activity with the condition without Vpr set to 100% (*P < 0.05).

Vpr weakly enhanced p21 promoter activity (Figure 6C). To examine whether Vpr suppress L1 retrotransposition in a p21-dependent manner, we used p21 knockdown 293T cells. Our results showed that Vpr could strongly suppress L1 retrotransposition even in p21 knockdown cells (Figure 6D and E), suggesting that Vpr suppresses L1 mobility in a p21-independent manner. To test whether Vpr and p21 affect L1 transcription and L1-encoded protein expression, we used an anti-human ORF1 antibody (SE-6798) (65). Vpr and p21 did not alter endogenous ORF1p expression (Figure 7A). Similarly, Vpr and p21 did not suppress ORF1p when expressed from plasmids pJM101/L1.3 and pJM105/L1.3, which respectively contain WT and ORF2p RT-mutant (D702A) human L1.3 element in a pCEP4 backbone vector (88) (Figure 7A and B). Moreover, Vpr did not enhance endogenous p21 expression (Figure 7A). To test the impact of Vpr on pCEP4 expression unit, we used the control backbone plasmid pCEP-GFP that expresses hrGFP (89). Vpr did not affect hrGFP expression from pCEP4 when both proteins were co-expressed in 293T cells (Figure 7B). Thus, Vpr and p21 did not affect L1 transcription and L1 ORF1p expression.

Vpr and p21 suppress L1 ORF2p-mediated reverse transcription

To examine whether HIV-1 Vpr interacts with L1 ORF1p and/or ORF2p, we performed immunoprecipitation and immunofluorescence studies. We did not observe a co-immunoprecipitation of Vpr with ORF1p (Figure 8A). ORF1p was found in cytoplasmic foci (Figure 8B), consistent with previous reports that ORF1p accumulates in cytoplasmic foci associated with stress granules (14,90). We did not observe co-localization of Vpr with ORF1p. In contrast, we noticed that Vpr and p21 co-immunoprecipitated with ORF2p by using TET-induced FLAG-tagged ORF2p (pLD401) (91) in TET293T cells (Figure 8C). In addition, we confirmed that p21 co-immunoprecipitated with FLAG-tagged ORF2p (pTM02F3) (89) in 293T cells (Figure 8D). Furthermore, we observed a partial colocalization of ORF2p with either Vpr or p21 (Figure 8E). Vpr alone
The level of L1 ORF1p in presence of Vpr WT and H71R mutations (Figure 8B), whereas Vpr partially colocalized with ORF2p predominantly localized to nucleus and nuclear membrane (Figure 8E). Thus, Vpr and p21 seem to interact with L1 ORF2p but not ORF1p, suggesting a possibility that Vpr and p21 inhibit L1 retrotransposition through ORF2p-mediated activities. To test this possibility, we examined whether Vpr and p21 suppress the L1 reverse transcriptase (RT) activity by LEAP assay. For this approach, we first enriched for L1 RNP from transfected 293T cell extracts, since ORF2p is characterized by a low level of expression due to its unconventional translation mechanism (14,92). L1 RNA assembles with ORF1p and ORF2p to form RNP complexes in the cytoplasm from which ORF2p RT activity can be detected. Indeed, we detected L1 ORF1p and ribosomal S6 proteins by western blot in the enriched RNP fractions (Figure 9A). S6 protein is abundant in cellular RNP preparations. Interestingly, we noticed that low but detectable amounts of Vpr and p21 were present in the RNP fractions when co-expressed with L1 (Figure 9A), suggesting that Vpr and p21 may be part of L1 RNPs. However, at this stage, the data presented are not sufficient to determine if these proteins actually form part of the L1 RNP. In this context, ORF2p RT activity measured by LEAP assay was significantly reduced with Vpr WT or p21 (Figure 9B), indicating that Vpr and p21 strongly inhibit the L1 RT activity. Consistent with the result of L1 retrotransposition with Vpr H71R (Figure 3E and F), Vpr H71R had a marginal effect on the ORF2 RT activity compared with that of Vpr WT (Figure 9B). Altogether, Vpr and p21 seem to restrict L1 retrotransposition through an inhibition of ORF2p RT activity.

DISCUSSION

Several host cellular factors have been involved in the regulation of L1 mobility (12,21–27,91,93). In this study, we have demonstrated that viral (Vpr) and cellular (p21 and p27) cell cycle regulators restrict L1 mobility. Both p21 and p27 belong to the Cip/Kip family of CDK kinase inhibitors (CKIs) (73–77). p21 is the first reported cell cycle inhibitor, blocking cell cycle at G1/S phase (73–75). p27 arrests the cell cycle at G1/S phase (76,77). Vpr arrests the host cell cycle at G2/M phase (53). Therefore, cell cycle control might be important for L1 retrotransposition. In this regard, Shi et al., previously reported that L1 retrotransposition was strongly inhibited in cells arrested at G1, S, G2 or M phase, indicating that cell division is required for L1 retrotransposition (94). However, the same study found that the levels of L1 transcripts were strongly reduced in arrested cells, suggesting that transcriptional inhibition is largely responsible for reduced retrotransposition observed (94). In contrast, both Vpr and p21 did not suppress L1 transcription (Figure 6A and B), indicating that Vpr and p21 inhibit L1 mobility post-transcriptionally. This result is consistent with studies reporting limited retrotransposition levels in cell cycle arrested cells that could not be explained by reduced L1 transcription (95,96). It suggests that even if retrotransposition can occur in arrested cells, cell division can promote L1 retrotransposition efficiency (95,96). This observation is in disagreement with other studies reporting evidence of retrotransposition in non-dividing cells (96,97).

On the other hand, Mita et al., reported that ORF1p together with ORF2p and L1 mRNA enters the nucleus during mitosis and retrotransposition appears to occur mainly during S phase, indicating a cell cycle bias for L1 retrotransposition (98). Furthermore, recent studies reported that ATM and p53, a master regulator of cell cycle, also regulate L1 mobility (99–101), even though we observed no effect of overexpression of ATM in L1 retrotransposition (Figure 4A). Interestingly, PCNA, the polymerase δ-associated sliding DNA clamp, interacts with L1 ORF2p through a PCNA in-

**Figure 7.** Vpr and p21 do not suppress L1 ORF1p expression. (A) The level of L1 ORF1p in presence of Vpr WT and H71R mutant. 293T cells (2 × 10^5 cells/well) were cotransfected with 2 µg of pCEP-GFP, pJM105/L1.3 reverse transcriptase-deficient mutant (88), or pJM101/L1.3 wild-type L1 (88), and 2 µg of pcDNA3-HA, pcDNA3-HA-Vpr WT, pcDNA3-HA-Vpr H71R, or pcDNA3p21. Cells were cultured for 4 days, lysed and subjected to western blot to analyze the expression of ORF1p using anti-hORF1P antibody (SE-6798) (65). Western blotting of the cell lysates with anti-Vpr, anti-p21, and anti-β-actin antibodies is also shown, respectively. (B) Protein expression level of L1 ORF1p and hrGFP in presence of Vpr WT and H71R mutant. 293T cells were transfected with pJM105/L1.3 or pCEP-GFP, together with either pcDNA3-HA or pcDNA3-HA-Vpr WT and cultured for the indicated time. Western blotting of the cell lysates with anti-hORF1p, anti-Vpr, anti-GAPDH and anti-hrGFP (240141; Agilent) antibodies is also shown, respectively.
Figure 8. HIV-1 Vpr and p21 bind to L1 ORF2p. (A) Vpr does not bind to L1 ORF1p. 293T cells (2 × 10^5 cells/well) were co-transfected with 2 μg of pcDNA3-HA-Vpr and 2 μg of pcDNA3-FLAG-ORF1. The cell lysates were immunoprecipitated with anti-FLAG (M2) antibody, followed by immunoblot analysis using anti-FLAG (M2) and anti-HA (HA-7) antibodies. (B) Subcellular localization of HA-ORF1p and EGFP-Vpr. 293T cells were co-transfected with pcDNA3-HA-ORF1 (100 ng) and pEGFP-Vpr (100 ng). Cells were stained with anti-HA (HA-7) antibody and then visualized with Cy3-conjugated anti-mouse antibody. Nucleus was stained with DAPI. Images were obtained using confocal laser scanning microscopy (FV1200, Olympus). (C) Vpr and p21 bind to ORF2p. TET293T cells (2 × 10^5 cells/well) were transfected with pLD401 (91). Cells were incubated with 1 μg/ml puromycin for 3 days and then treated with 500 ng/ml doxycycline for 2 days. Then, the cells were transfected with either pcDNA3-HA-Vpr or pcDNA3p21. 2 days post-transfection, the cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblot analysis using anti-FLAG, anti-HA, or p21 antibody. (D) p21 binds to ORF2p. 293T cells (2 × 10^5 cells/well) were transfected with pTM02F3 (89). Three days post-transfection, the cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblot analysis using anti-FLAG and p21 antibodies. (E) Subcellular localization of Vpr, p21, and/or ORF2p-FLAG. 293T cells were co-transfected with pTM02F3 (100 ng) and either pcDNA3-HA-Vpr (100 ng) or pcDNA3 p21 (100ng). Cells were stained with anti-FLAG (M2) and either anti-HA (3F10) or anti-p21 antibodies and then visualized with Alexa596-conjugated anti-mouse and either Alexa488-conjugated anti-rat or anti-rabbit antibodies. Nucleus was stained with DAPI. Images were obtained using confocal laser scanning microscopy.

The interaction of ORF2p with PCNA is critical for L1 retrotransposition (91). In this regard, PCNA is known to bind to the C-terminal domain of p21 (56), suggesting a possibility that p21 competes with L1 ORF2p for the PCNA-binding, resulting in the repression of L1 retrotransposition. However, the C-terminal half of p21, p21C, did not reduce L1 retrotransposition (Figure 3C and D), indicating that p21 restricts L1 retrotransposition in a PCNA independent manner. In this study, we showed that p21 interacts with L1 ORF2p and inhibits the ORF2p reverse transcriptase activity in LEAP assay (Figures 8 and 9).

We showed that the inhibitory effect of a mutant Vpr (H71R), which is unable to arrest the cell cycle, was significantly reduced compared with that of wild-type Vpr (Figure 3E and F), suggesting that Vpr suppresses L1 mobility in a cell cycle dependent manner. Independent of cell cycle control, we have demonstrated that Vpr restricts L1
Vpr and p21 suppresses L1 reverse transcriptase activity. (A) Western blotting of human ORF1p, HA-Vpr, p21 or S6 protein as a loading control in RNP prepared from 293T cells transfected with the indicated plasmids: pCEP4 and pcDNA3-HA (HA) empty vectors (–), pJM105/L1.3 and pcDNA3-HA (-) or pJM101/L1.3 together with pcDNA3-HA (-), pcDNA3-HA-Vpr WT, pcDNA3-HA-Vpr H71R, or pcDNA3 p21. (–) Detection of L1 RT activity by LEAP assay (top panel) and of L1 RNA (middle panel) or GAPDH RNA (bottom panel) by RT-PCR. LEAP assay was performed with RNP prepared from cells transfected with indicated plasmids. PCR control denotes a control for the PCR step without cDNA. LEAP/RT control denotes a control for RT step without RNP. RNP samples used in panel A and B are from the same replicate. The experiment was replicated three times with similar results.

retrotransposition by inhibiting ORF2p-mediated reverse transcriptase activity (Figure 9B). Moreover, despite limited levels of ORF2p detection, possibly due to unconventional translational mechanism (14,92) or protein stability, we found that Vpr communoprecipitates with L1 ORF2p but not ORF1p (Figure 8A, C and D). Vpr can also be detected in cellular RNP fractions alongside with L1 (Figure 9A), providing additional circumstantial evidence that Vpr associates with ORF2p and inhibits RT activity (Figure 9B). On the other hand, p21 has been involved in restriction of HIV-1 during reverse transcription (102–105). Ribonucleotide reductase (RNR) increases the dNTP pool and HIV-1 relies on cellular dNTPs for its reverse transcription. p21 represses dNTP biosynthesis by down-regulating the expression of the RNR2 subunit of ribonucleotide reductase, resulting in the restriction of HIV-1 (104). Furthermore, Pauls et al. recently reported that p21 regulates a sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD1) (105). The deoxynucleoside triphosphohydrolase SAMHD1 has been identified as a novel myeloid cell specific HIV-1 restriction factor (106–111). Indeed, SAMHD1 inhibits HIV-1 reverse transcription by depleting the intracellular dNTP pool (111) and restricts L1 retrotransposition by inhibiting the ORF2p-mediated L1 reverse transcription (112). Similarly, we have demonstrated that p21 interacts with ORF2p (Figure 8C and D) and can also be detected in cellular RNP fractions alongside with L1 (Figure 9A), resulting in inhibition of ORF2p RT activity (Figure 9B). Therefore, in HIV-1-infected patient cells or tumor cells with overexpression of p21, or a mutation in the N-terminus of p21 or p27, these cell cycle regulators may potentially impact L1 retrotransposition. However, if p21 is overexpressed in HIV-1-infected cells, p21 may inhibit both HIV-1 and L1 mobility. As Vpr is known to enhance the p21 promoter activity (86,87), it raised the possibility that Vpr transcriptionally induces the p21, resulting in the suppression of L1 mobility. However, we showed that Vpr did not accumulate endogenous p21 expression in 293T cells (Figure 7A) and Vpr could suppress L1 retrotransposition even in p21 knockdown cells (Figure 6D and E), suggesting that Vpr suppresses L1 mobility in a p21-independent manner.

Since endogenous retroelements such as L1, Alu, SVA, and HERVs are abundant and constitute ~45% of the human genome, they may influence the integrated exogenous HIV-1 retroviruses, or vice versa. In this study, we demonstrated that L1 ORF1p is incorporated into the HIV-1 virions (Figure 5), suggesting a possibility that infectious HIV-1 particle might horizontally transmit L1 into target cells as a vector. Another possibility is that the incorporated ORF1p affects the HIV-1 infectivity. However, the incorporation of ORF1-HA into HIV-1 virion did not affect the HIV-1 infectivity, except only HA-ORF1 moderately suppressed the infectivity of NL4-3 but not JR-FL (Figure 5C and D), even though similar amounts of ORF1p were incorporated into both NL4-3 and JR-FL virion (Figure 5A). Since ORF1p is an RNA-binding and nucleic acids chaperone protein, we also examined whether ORF1p modulates the HIV-1 Tat-mediated HIV-1 transcription or HIV-1 Rev-dependent nuclear export function of HIV-1 RNA. However, ORF1p did not affect either Tat or Rev function (Figure 5E and F), suggesting that ORF1p might not bind and affect HIV-1 RNA. In this regard, we failed to observe the interaction of ORF1p with HIV-1 RNA (data not shown). Furthermore, we found that the incorporation of ORF1p into HIV-1 virions was independent of HIV-1 accessory proteins including Nef, Vpr, Vpu and Vif, because the mutant viruses lacking the expression of each viral gene still retained the ability to incorporate ORF1p (Figure 5B). Since it has been indicated that several P-body and stress granule components, such as APOBEC3G, APOBEC3F and MOV10, are incorporated into HIV-1 virions through an interaction with nucleocapsid protein (NC) (78–82), ORF1p, a component associated to stress granule
(14,90), also might be incorporated into virion through an interaction with NC. To fully explain these phenomena, further mechanistic studies are required. Jones et al. demonstrated that the L1 DNA accumulates in the HIV-1 infected cells (113). In this regard, we also observed that extracellular Vpr protein in the culture medium induces L1 retrotransposition (114). In contrast, we found that Vpr strongly suppresses L1 retrotransposition when co-expressed in the cell (Figure 2). The cause for this discrepancy may be due to technical differences and experimental strategies. Iijima et al. only used recombinant Vpr protein in their experiments. In contrast, we used Vpr-expressing plasmids to obtain intracellular expression of Vpr in dividing cells. All tested plasmids (pcDNA3-HA-Vpr, pCMX Vpr96, pAIP-Vpr and pEGFP-Vpr) could strongly suppress L1 retrotransposition and LEAP assay showed that Vpr inhibits L1 RT activity (Figure 9B). Except for the demonstration that Vpr inhibits reverse transcription by ORF2p, the other proteins may be inhibiting L1 indirectly or act on cell cycle to limit the expression of the reporter cassette.

Finally, if L1 actively jumps and inserts a new copy of L1 in the integrated HIV-1 proviral locus or in genes essential for the infected cell survival, it will result in the destruction of HIV-1 or the infected cell. Therefore, we can speculate that HIV-1 may restrict L1 mobility to protect its own genome and the genome of the infected cell from de novo L1 insertional mutagenesis. Altogether, we suggested that viral and cellular cell cycle regulators can negatively impact L1 mobility through an inhibition of ORF2p reverse transcriptase.

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