Cell–cell and virus–cell fusion assay–based analyses of alanine insertion mutants in the distal α9 portion of the JRFL gp41 subunit from HIV-1

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Running title: Alanine insertion mutants in JRFL gp41 α9

ABSTRACT

Membrane fusion is the first essential step in HIV-1 replication. The gp41 subunit of HIV-1 envelope protein (Env), a class I fusion protein, achieves membrane fusion by forming a structure called six-helix bundle (6HB) composed of N- and C-terminal heptad repeats (NHR and CHR, respectively). We have recently shown that the distal portion of the α9 helix in CHR of X4-tropic HXB2 Env plays a critical role in the late-stage membrane fusion and viral infection. Here, we used R5-tropic JRFL Env and constructed six alanine insertion mutants, 641+A to 646+A, in the further distal portion of α9 where several glutamine residues are conserved (the number corresponds to the position of the inserted alanine in JRFL Env). 644+A showed the most severe defect in syncytia formation. Decreased fusion pore formation activity, revealed by a dual split protein assay, was observed in all mutants except 641+A. Sequence analysis and substitution of inserted 644A with Q revealed that the glutamine residue at position 644 that forms complex hydrogen bond-networks with other polar residues on the surface of 6HB is critical for cell-cell fusion. We also developed a split NanoLuc (Nluc) reporter-based assay specific to the virus-cell membrane fusion step to analyze several of the mutants. Interestingly syncytia-competent mutants failed to display Nluc activities. In addition to defective fusion activity, a reduction of Env incorporation into virions may further contribute to differences in cell-cell and virus-cell fusions.

HIV-1 is a member of the enveloped viruses and it initiates its lifecycle with membrane fusion between the virus and cellular membranes (1,2). This essential step begins with the interaction between the gp120 subunit of the viral envelope protein (Env) and the cellular receptor/co-receptor complex (CD4 and chemokine receptors). The step of the actual membrane fusion is mediated by the gp41 subunit of Env, a member of the class I fusion proteins.

The gp41 subunit undergoes drastic conformational changes during membrane fusion to form a six-helix bundle (6HB) structure (3) (Fig. 1A). The 6HB is generated by the anti-parallel packing of two helices of gp41...
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termed as N- and C-terminal heptad repeats (NHR and CHR, respectively) (4,5). The core of 6HB is composed of a parallel coiled coil of three NHRs, and CHRs bind to the grooves generated on the trimeric NHR bundle (Fig. 1B). The formation of 6HB is tightly linked to the fusion pore formation, its enlargement, and eventual merging of the virus and cell membrane (6,7). Since the inhibition of 6HB formation results in the abortion of membrane fusion (8,9), extensive studies of 6HB such as alanine scanning mutagenesis have been conducted in the past(10-13); however, the exact physical location and the mechanism of the potential association of 6HB with the fusing lipid bilayers are unknown, and the exact mechanism of pore enlargement during infection remains elusive.

In a previous study, we reported alanine insertion mutants in the HXB2 gp41 subunit (14). We found that the mutants within α9 following its exit from the bottom of the four-helix collar formed by α6, α7, α8, and α9 (3) did not affect Env biosynthesis but showed impairment in membrane fusion. In a cell-cell fusion assay, a certain insertion mutant in the proximal region of α9 was able to form fusion pores as measured by the split reporter protein called DSP (dual split protein) (15,16); however, it failed to support viral infection in a conventional viral infection assay relying on the expression of the packaged reporter gene. Viral infectivity was recovered when insertion sites were shifted toward the distal portion of α9.

Our data are consistent with the preceding studies indicating that 6HB formation may start from the proximal portion of CHR and that fusion pores made before the completion of 6HB formation may be stabilized and enlarged along with the progress of 6HB formation toward the distal portion of CHR (6,7). The conventional virus-cell fusion assay used in our previous study has a technical caveat: the reporter expression requires multiple steps after the actual membrane fusion step such as reverse transcription, integration, transcription, and translation of the reporter gene. In this study, to measure the virus-cell fusion more specifically independent of these downstream steps, we developed a new split NanoLuc luciferase (Nluc)-based (17,18) virus-cell fusion assay in which a small peptide called HiBIT derived from Nluc was targeted into virions via fusion with the viral protein, Vpr (19,20). These engineered virus-like particles (VLPs) were used to infect target cells expressing the rest of Nluc called LgBiT. We named this new assay R-BiT (Vpr-HiBiT) assay. R-BiT assay can be completed in an hour after infection.

Here, we used JRFL Env and extended our alanine insertion mutagenesis of gp41 to further downstream of α9 of CHR where several glutamine residues are conserved between HXB2 and JRFL. We found that a conserved glutamine residue at position 644 (based on JRFL Env numbering) is critical for cell-cell membrane fusion especially in syncytia formation. With our new R-BiT assay, we found that the mutants that were relatively efficient in cell-cell fusion activities showed severe defects in virus-cell membrane fusion and that the reduction of Env incorporation into virions in addition to the defective fusion activity may contribute to the defective virus-cell fusion of the mutants.

RESULTS

Effect of alanine insertion in the distal portion of α9 in JRFL CHR on cell-cell fusion activity

Extending our previous study of HXB2 Env, we generated alanine insertion mutants in the distal portion of α9 using R5-tropic JRFL Env. The primary structures of HXB2 and JRFL gp41 including NHR and CHR are shown in Fig. 2A. Similar to an authentic homotrimeric coiled coil (21), the positions a and d of gp41 NHR are often occupied by hydrophobic residues such as I and L, but the presence of polar residues such as Q are also noted. Intriguingly, there were Q triplets around residue 541 of JRFL gp41 and 550 of HXB2 Env (note, HXB2 number can be obtained by adding 9 to that of JRFL Env) (Fig. 2A).
Although it does not form a homotrimeric coiled coil like NHR, CHR is also given an arbitrary \((abcdefg)n\) representation; the CHR residues in positions \(a\) and \(d\) are expected to interact with the \(e\) and \(g\) residues of NHR (Fig. 1B, right) and contain some hydrophobic residues. The frequency of hydrophobic residues is, however, less in the latter half of \(\alpha 9\) where JRFL and HXB2 have identical sequences (Fig. 2A). In contrast to this conservation, the proximal half of \(\alpha 9\) of JRFL and HXB2 shows several differences, though conservative in its nature.

We chose the region 641 to 646 of \(\alpha 9\) for our alanine insertion mutagenesis (indicated by the arrow heads in Fig. 2A) based on two reasons. First, this region contains multiple polar Q residues and forms complex hydrogen bond-networks in 6HB by interacting with the NHR region, which is also rich in Q residues (4,5) (Fig. 1B, left and middle). Second, as shown in our previous study (14), mutations in this region of \(\alpha 9\) are less likely to affect the synthesis and processing of gp160 to compromise functional analysis since it is far from the cleavage site between gp120 and gp41 in the native structure (3). The mutants were named based on the position of the inserted alanine residue in the JRFL Env. For example, for the 641+A mutant, the alanine residue is located at position 641.

We used a codon-optimized synthetic JRFL Env gene (22) to express envelope proteins. We first examined the syncytium formation activities of the mutants by transfecting expression vectors into CD4+CCR5+ cells. Mutant 644+A showed a very severe defect in this assay (Fig. 3 A and B). Though attenuated in some mutants, syncytia formation activity was retained in other mutants. Although we reasoned that mutations in this region would not perturb the protein biosynthesis and processing of Env as discussed above, we next analyzed the protein profile of 644+A and other mutants. The immunoblotting result with an anti-gp120 antibody failed to reveal severe defects in expression or processing in 644+A (Fig. 3C), suggesting that 644+A has a defect in the membrane fusion step itself.

To gain further insight into the potential step(s) accounting for the defect in the cell-cell membrane fusion of 644+A, we used the DSP assay to test whether 644+A has a defect in fusion pore formation. The DSP assay detects the communication (pore formation) between effector cells and target cells by measuring the recovery of the Renilla luciferase activities of split DSPs (23). The result is shown in Fig 3D. 644+A showed the lowest DSP activity corresponding to about 25% of that of the wild type. This suggests that mutant 644+A has defects in pore formation as well as the subsequent steps such as pore enlargement that eventually leads to syncytia formation. The other mutants showed decreased DSP activities, but 641+A showed a similar DSP activity to that of the wild type. Overall, there is a good correlation between syncytia formation and DSP assays.

The glutamine residue at position 644 plays a critical role in membrane fusion

An alanine insertion can have more detrimental effects than a simple alanine substitution by inducing a shift of amino acid residues after the insertion point. However, previous studies suggested that such a mutation can be accommodated even in an \(\alpha\)-helix (24,25). Since mutants 641+A, 642+A, and 643+A showed a relatively mild defect in syncytia formation, it can be suggested that the \(\alpha 9\) region after residue 641 is rather tolerant to shifting by insertion. Consistent with this idea, mutants 645+A and 646+A retained cell-cell fusion activities. This led to the question why only mutant 644+A showed such a severe defect in syncytia formation.

When we aligned the mutant amino acid sequences, it became clear that only mutant 644+A differed from the others by having an alanine residue at position 644 (Fig. 2B). This alteration of Q at position 644 did not occur in mutants 641+A, 642+A, and 643+A because of the presence of the Q residue at position 643;
that is, Q643 was shifted to position 644 by alanine insertion in these mutants. Similarly, mutants 645+A and 646+A did not show alteration of 644Q. This suggests that the loss of the Q residue at position 644 may be responsible for the observed severe defect in the membrane fusion of 644+A. To test this hypothesis, we mutated the inserted alanine of 644+A to Q; this has been shown as 644+Q in Fig. 2B because it is equivalent to the insertion of Q at position 644. We analyzed the phenotype of 644+Q. As shown in Fig. 3, improved syncytia formation and DSP activity were observed, suggesting that the presence of a Q residue at position 644 is critical for Env function in mediating cell-cell membrane fusion.

**Analysis of alanine insertion mutants in virus-cell fusion assay; Development of a virus-cell fusion assay (R-biT assay) by employing the split Nluc system**

Next, we intended to examine these mutants in a virus-cell fusion assay since we and others have observed some discrepancies between the cell-cell fusion assay and virus-cell fusion assay (10,14,26). For this purpose, we tried to develop a more fusion-specific virus-cell fusion assay using split Nluc as a reporter. Split Nluc recovers its activity via self-association of the Nluc-derived small peptide (HiBiT) and the remaining domain of Nluc (LgBiT). Our approach is similar to the BlaM assay (27). HiBiT was targeted into HIV-1 virions via the virion-associated viral protein, Vpr (19,20).

Accordingly, we named this assay as R-BiT (Vpr-HiBiT) assay. Nluc is more sensitive than Renilla luciferase and its signal detection is simple and does not require image analysis like the BlaM assay. In our pseudotype-based assay, we also added the packageable reporter gene (firefly luciferase) used in our previous study (14) for comparison (supplemental Fig 1). The 293 cells stably expressing CD4, CCR5, and LgBiT were used as target cells (see Materials and Methods section).

In our initial attempt of the JRFL Env-based assay, we encountered a relatively high Nluc background signals in the presence of a CCR5 antagonist, maraviroc (MVC) (Fig. 4A), while the Fluc reporter signal was efficiently blocked (Fig. 4C). The addition of the membrane-impermeable DrkBiT peptide (about DrkBiT, see the experimental procedures section below), a competitive peptide against HiBiT, that binds to LgBiT without recovering the Nluc activity reduced these background signals (Fig. 4A), and the signal to background ratio was dramatically improved (Fig. 4B). DrkBiT did not affect Fluc reporter signals (Fig. 4C). These data suggested that reassociation of the leaked Vpr-HiBiT and/or LgBiT was responsible for the observed residual background.

**Modification of LgBiT to reduce the background signal**

To reduce the background, we tried to minimize the spontaneous leakage of LgBiT from the target cells as the sedimentation of virions by ultracentrifugation did not eliminate the background completely (data not shown). We engineered LgBiT by fusing betagalactosidase (BetaGal-LgBiT), a phosphatidylinositol-binding pleckstrin homology (PH) domain (PH-Halo-LgBiT) (28), and a farnesylation motif (HCAAAX) (29) to LgBiT to reduce the leakage of LgBiT (Halo-LgBiT-HCAAAX). The results shown in Fig. 4D showed that PH-Halo-LgBiT, betaGal-LgBiT, and Halo-LgBiT-HCAAAX decreased the MVC-insensitive background signals compared with the unmodified Halo-LgBiT. Comparison of Nluc activities between DMSO and MVC showed that the addition of the PH domain yielded the best reduction of the background signal (Fig. 4E). The modification of LgBiT did not affect Fluc reporter signals (Fig. 4F). The degree of leakage estimated by the ratio of the extracellular and intracellular amounts of LgBiT was high for beta-galactosidase and HCAAAX. This ratio was better for PH than for beta-galactosidase and HCAAAX (Fig. 4G). Accordingly, we used the target cells expressing PH-Halo-LgBiT in the subsequent experiments.
Importance of a polar residue in gp41 α9 in membrane fusion

Specificity of the R-BiT assay for membrane fusion

The R-BiT assay was validated for its specificity for membrane fusion by neutralizing antibodies (2G12 and PGT151) (30,31) or an RT inhibitor (azidethymidine; AZT). The addition of neutralizing antibodies significantly reduced the Nluc signals (Fig. 5A). As expected, AZT did not affect Nluc signals (Fig. 5A). However, after 24 h of infection with no detectable residual signals of Nluc (Fig. 5B), the Fluc activities expressed from the integrated provirus DNA were inhibited by all inhibitors (MVC, 2G12, PGT151, and AZT) (Fig. 5B). These results confirmed that our R-BiT system could faithfully monitor the membrane fusion step of viral infection. As a byproduct of the incorporation of Vpr-HiBiT into virions, we found that the amount of HIV-1 virions can be estimated by titrating the virion-associated Vpr-HiBiT. Nluc signals have a wider dynamic range than the p24 assay and the determination can be completed in 1 h (supplemental Fig 2).

Application of the R-BiT assay to the analysis of gp41 mutants

We tested mutants 641+A, 644+A, and 644+Q with our new R-BiT assay. We added the dose-adjusted VLPs to the target cells (293CD4R5-PH-Halo-LgBiT cells), and the Nluc activity was monitored (Fig. 6A). The wild type showed a time-dependent large increase in Nluc activity, but all the tested gp41 mutants showed almost no Nluc activity. This suggests that these mutants have a severe defect in the virus-cell membrane fusion step. The loss of infectivity for mutant 644+A was expected because it failed to develop syncytia in our cell-cell fusion assays. The results of 641+A and 644+Q were, however, rather unexpected especially because the syncytia formation and DSP activities of the 641+A and 644+Q were almost similar to those of the wild type (Fig. 3). This consistent result was obtained with the conventional virus infection assay relying on the expression of the packaged reporter gene (Fig. 6B). Our data revealed that there are prominent differences in fusion phenotypes between cell-cell and virus-cell membrane fusions. All the other mutants also showed no Fluc activities (Fig. 6B).

To determine the reason for the observed differences in the cell-cell and virus-cell fusion assays, we analyzed the protein profiles of the virions (Fig. 6C). When the gp41/p24 ratio was calculated, the mutants exhibited about 30% reduction of the incorporation of processed Env compared with the wild type. Furthermore, a decreased gp120/p24 ratio was observed for all the mutants, indicating a gp120 shedding for the mutants (Fig. 6C). This gp120 shedding may further contribute to the observed differences in cell-cell and virus-cell fusions. In addition to these differences, we also noted increased incorporation of gp160 into mutant virions when the bands recognized by anti-gp41 were used to calculate the gp160/p24 ratio. This tendency was less prominent when the bands recognized by anti-gp120 were used. We cannot rule out the possibility that this could arise from the differences in the recognition of the antigens by the different antibodies. The presence of gp160 on the virions may interfere with the receptor recognition by the processed gp120. In addition to the defective fusion activity, these changes in viral protein profiles of the mutants may contribute to their severe defects in virus-cell fusion.

DISCUSSION

In this study, we performed alanine insertion mutagenesis in the distal region of α9 in JRFL gp41. Most of our alanine insertion mutants retained syncytia formation activity except for 644+A. The 644+4A mutant showed attenuated activities in fusion pore formation, as revealed by the DSP assay. Therefore, mutant 644+A seems to have more severe defects in membrane fusion from pore formation to the progress of membrane fusion after fusion pore formation.

Our data suggest that the presence of a Q residue at position 644 is critical for cell-cell membrane fusion. The results of a previous alanine substitution (not an insertion) study of
the 644Q residue in JRFL (10) showed approximately 50% reduction in cell-cell and virus-cell fusion and support the importance of 644Q in membrane fusion. We observed more severe defects in both assays, and this is probably due to the more destructive nature of alanine insertion than alanine substitution.

Since an alanine insertion causes deregistration of amino acid residues downstream of the insertion point, it is expected that an insertion will have a stronger negative effect in the 6HB structure where multi-helix interactions are involved. In contrast to this expectation, though attenuated, most of the mutants retained syncytia formation activity except for 644+A. We speculate that the tolerance to insertion in this region could be due to the presence of multiple polar residues, such as Q, near the insertion sites. Polar residues such as Q are known to destabilize the coiled coil (32) and this may provide some flexibility or plasticity in these α-helix regions. An insertion of A or Q could be accommodated by local underwinding of the helix with associated changes in superhelix to accommodate underwinding of the involved helices (33). Even with this underwinding effect, there was a striking difference between 644+A and 644+Q in cell-cell membrane fusion.

Johnson et al. have shown that the contribution of the latter one third of 6HB where residue 644 resides for the stability of 6HB is modest (34), and this agrees with the data of Suntoke et al. who did not observe any severe impact of Q653A mutation (corresponds to 644Q in JRFL) on the stability of 6HB (35) in HXB2 Env. Although we cannot tell whether the result of HXB2 is immediately applicable to JRFL, and the stability of 6HB in our 644+A or Q needs to be determined in the future, our data suggest that the chemical nature of the amino acid residues at position 644 and its relationship with the polar residues in the exposed surface of 6HB may play some roles during membrane fusion in addition to the 6HB stability.

Regarding the residue at position 644 in JRFL 6HB, Q644 (Q653 in HXB2) located at position e of the helix of CHR interacts with 544N (N553 in HXB2) at position b of NHR of the same gp41 molecule (see Fig 1B, right) (4,5). In this layer of 6HB, 644Q is involved in complex hydrogen bonding networks formed among several Q and N residues of both NHRs and CHRs (5,35). Interestingly, there are 5 or 6 Q residues clustering on the surface of 6HB. The substitution of 644Q with A affects the hydrogen bonding and clustering of Q residues. Even though the exact mechanism remains to be elucidated, the involvement of Q-rich proteins in protein aggregation (36,37) may suggest that clustering of multiple Q residues on the surface of 6HB may affect the manner of interaction of 6HB with other viral or cellular (membrane) protein(s) during membrane fusion. This may affect the fate of membrane fusion. Further mutagenesis of 644Q or neighboring polar residues with other amino acid residues may provide an insight into the underlying molecular mechanism.

We have developed a new Nluc-based virus-cell fusion assay and observed a discrepancy in membrane fusion efficiency between the cell-cell and virus-cell fusion assays; cell-cell fusion was more tolerant to mutations. In our previous study of HXB2 Env, we observed a similar discrepancy and raised the possibility that the insufficient fusion pore size generated by the mutant is responsible (14). This insufficient size of fusion pores may be able to explain the severe defect of mutant 644+A, which failed to form syncytia. However, our reporter for the R-BiT assay was a small virion-associated protein, Vpr-HiBiT, which could be more easily released from virions upon membrane fusion and subsequent disassembly of viral cores. Since we failed to observe virus-cell fusion with the R-BiT assay for the mutant, 644+Q, which recovered the cell-cell fusion activity, the insufficient size of fusion pores alone may not be able to account for the observed differences in the other mutants.
Importance of a polar residue in gp41 a9 in membrane fusion

In addition to the defective fusion activities of mutant Env, we cannot rule out the possibility that the difference in the number of available functional Envs on the cell or virus surface in a given time period may affect the outcome. While a virus has no additional supply of new Envs during membrane fusion, newly synthesized Envs can be recruited in cell-cell fusion during the period of membrane fusion since protein synthesis is continuous during cell-cell fusion. We observed a reduction in the incorporation of the processed Env into virions accompanied with gp120 shedding. Furthermore, as described for JRFL Env previously (10), indiscriminate incorporation of unprocessed gp160 into virions was noted. These may contribute to the observed inefficient virus-cell fusion. It seems that mutations in the distal portion of a9 can still affect the incorporation of Env into virions in JRFL Env.

Another possibility is the involvement of cellular proteins in the process of membrane fusion. The contribution of adhesion molecules such as LFA-1 and ICAMs to HIV-1 Env-mediated cell-cell fusion has been described (38,39). Although these proteins are absent in our 293-derived effector and target cells, the involvement of some other cellular proteins may facilitate cell-cell fusion. The alterations of 6HB surface by our mutation may affect the cell-cell fusion by altering the interactions between 6HB and cellular proteins.

Lastly, our new Nluc-based virus-cell fusion assay, R-BiT, is easier to perform and is faster for monitoring virus-cell fusion in a fusion-specific manner independent of downstream replication cycles such as reverse transcription/integration, and thus, will be a useful tool for investigating these potential differences between cell-cell and virus-cell fusions.

**EXPERIMENTAL PROCEDURES**

**Cell lines, chemicals, antibodies, transfection**

293-CD4-CCR5 (293CD4R5), LgBiT-expressing 293CD4R5, and 293T-HiBiT cell lines, generated and used in this study (see below), were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Wako, Japan) containing 10% fetal bovine serum (FBS). For the establishment of stable cell lines expressing CCR5, HiBiT, and each LgBiT protein, recombinant pseudotyped retroviruses were produced using plat-E cells (kindly provided by Professor Toshio Kitamura, Institute of Medical Science, University of Tokyo, Tokyo, Japan (40) with a vesicular stomatitis virus (VSV)-G-expressing plasmid (pCMV-VSV-G). The cells infected with pseudotyped viruses were selected with 1 µg/mL puromycin and/or 10 µg/mL blastcidin for at least 1 week. These bulk selected cells were used to perform fusion assays. Maraviroc (Cayman Chemical Company, Ann Arbor, MI, USA) was dissolved in DMSO at a concentration of 0.1 mg/ml. DrkBiT peptide (with the VSGWALFKKIS sequence, kindly provided by Promega, Madison, WI, USA through personal communication) was dissolved in H2O at a concentration of 0.1 mg/ml (41). AZT (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in phosphate-buffered saline [PBS(–)] at a concentration of 0.1 mM. Human IgG1 isotype control (MBL, Nagoya, Japan), anti-gp120 neutralizing antibody C2G12 (Polymun Scientific, GmbH, Vienna, Austria), and PGT151 (Creative Biolabs Inc., Shirley, NY, USA) were dissolved in PBS (–) at a concentration of 200 µg/ml. For transient transfection, 293FT-HiBiT cells were transfected with pCMV-JRFLenv vector and TurboFect (Thermo Fisher Scientific, San Jose, CA, USA) according to the manufacturer’s instructions.

**Construction of expression vectors**

A synthetic DNA corresponding to the vpr gene of HIV-1 (42) was fused with HiBiT sequence (5'-GTG AGC GGC TGG CGG CTG TTC AAG AAG ATT AGC TAA-3') at the C-terminus by PCR and cloned into the pCMV vector. For the establishment of stable cell lines expressing CCR5, HiBiT, and each LgBiT protein, CCR5 was cloned into a pMXs-IRES- blastcidin retroviral vector, and Halo-HiBiT, LgBiT with β-galactosidase at the N-terminus,
and Halo-LgBiT with a PH domain at the N-terminus were cloned into the pMXs-Puromycin vector, respectively. For the establishment of Halo-LgBiT with H-Ras CAAX (HCAAX) at the C-terminus, the following sequences corresponding to the CAAX domain were fused at the C-terminus by PCR using KOD-Plus-Neo (Takara Bio, Otsu, Japan) followed by cloning into the pMXs-Puromycin vector. HCAAX: 5′-GAG TCC GGC CCC GGC TGC ATG TCC TGC AAG TGC GTG CTG TCC TAA-3′; KCAAX: 5′-AAG AAG AAG AAG AAG AAG TCC AAG ACC AAG TGC GTG ATC ATG TAA-3′; and QCAAX: 5′-CAG CAG CAG CAG CAG CA G TCC AAG ACC AAG TGC GTG ATC ATG TAA-3′. The gp41 CHR mutants were generated using an Env-expression vector containing a previously described codon-optimized JRFL sequence for mammalian cells (22). Complementary oligonucleotide pairs containing an insertion codon for the alanine residue, GCA, were used for mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

**Virus preparation**

For the preparation of Vpr-HiBiT containing lentiviruses, a plasmid mixture [2.5 µg pCMV-JRFLenv (JRFL-Env expression vector), 2.5 µg psPAX2 (Gag-pol, Rev, and Tat expression vector), 5 µg pLenti-CMV-Luc2 (packaging reporter to express f-luc), and 1 µg pCMV-Vpr-HiBiT (Vpr-HiBiT expression vector)] were transfected into 293T cells by calcium phosphate transfection. At 48 h after transfection, the supernatant was harvested and purified by ultracentrifugation (1 × 10⁵ g for 35 min using a TLA100.3 rotor). The pellet was then resuspended using HANK’s buffer. Viral lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (see below).

**R-BiT assay in a 384-well format**

A day before the R-BiT assay, the target cells expressing CD4, CCR5, and LgBiT were seeded in 12-well cell culture plates (2 × 10⁵ cells/ml). At 2 h before the assay, cells were treated with EnduRen (6 µM EnduRen; Promega), a substrate for NLuc, to activate EnduRen. MVC (1 µl) in DMSO, AZT, and neutralizing antibodies in PBS (−) were added to 384-well plates (Greiner Bioscience, Frickenhausen, Germany). Target cells were harvested by pipetting, spun down, and resuspended in DMEM containing 10% FBS and 6 µM EnduRen (8 × 10⁵ cells/ml). Next, 50 µl of single cell suspension and Vpr-HiBiT containing a dose-adjusted pseudotyped virus (70 ng of p24) with 10 µg/ml polybrene and 6 µM EnduRen were mixed in the wells. After incubation at 37 °C for 15, 30, and 60 min, light signals derived from HiBiT/LgBiT association were measured using CentroXS3 (Berthold Technol., Bad Wildbad, Germany). After incubation at 37 °C for 24 h, the supernatant was removed and Steady-Glo Luciferase assay reagent (Promega) was added to dissolve cells to measure Fluc activities.

**Cell-cell fusion assay - syncytium formation assay**

For the syncytia formation assay, expression vectors for Env were transfected into 293CD4R5 cells using TurboFect (Thermo Fisher Scientific, San Jose, CA, USA) and then incubated at 37 °C for 12-18 h. To visualize the nuclei of the cells, Hoechst 33,342 (5 mg/mL; Invitrogen) was applied at 37 °C for 15 min. After labeling, images were captured using a Fluorescent Inverted microscope (Olympus IX71). The number of nuclei in syncytia was divided by the total number of nuclei in the field to estimate the degree of syncytia formation. Two randomly chosen fields containing about 500 nuclei were examined.

**Cell-cell fusion assay – dual split protein (DSP) assay**

A cell-cell fusion assay using a split reporter protein termed DSP was performed as described previously (14). Briefly, the 293FT/DSP1-7 cells seeded in a 12-well plate (BD Falcon, San Jose, CA, USA) at a density of 3.0 × 10⁵ cells/well were transfected with Env-expression vectors of interest using FuGENE HD (FuGENE HD [µl]: DNA [µg]: Opt MEM [µl] = 2.5:1:50). Twenty-
four hours after transfection, the effector cells were mixed with the target cells in a 96-well View Plate (PerkinElmer) in triplicate. The target cells (293CD4R5/DSPc311) were plated in a 6-cm dish (BD Falcon, San Jose, CA, USA) at a density of 1.5 × 10^6/dish two days prior to the assay and were preincubated for 1 h with fresh medium containing 60 µM EnduRen (Promega). Membrane fusion was initiated by spinning down the mixed cells in a 96-well plate (130 g 1 min in LX-131; TOMY SEIKO CO., Ltd) at room temperature. The light signals derived from the reassociated DSP were measured using a GloMax-Multi Plus Detection System (Promega). A portion of the effector cells used for the DSP assay was used for CELISA to determine the surface level of Env as described previously (14). The cells were spun down in a collagen-coated 96-well plate (CORNING) (130 g for 3 min at room temperature). The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 5 min. After washing with PBS, endogenous peroxidases were inactivated with 3% hydrogen peroxide in PBS for 10 min. After blocking with a blocking reagent (5% ECL Prime Blocking Reagent (GE Healthcare, UK) in PBS) for 30 min, cells were incubated with a saturating amount of 2G12 (13.1 µg/mL, 1:1000 dilution in 0.5% ECL Prime Blocking Reagent) for 1 h at room temperature, and then incubated with anti-human IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) was used as the secondary antibody. The blot was further treated with an ECL Western Blot Kit (CWBIO, Beijing, China). Images were obtained using a LAS3000 system (Fujifilm, Tokyo, Japan).

**HiBiT Lytic assay and p24 ELISA**

For the detection of Vpr-HiBiT or LgBiT, 25 µl of viruses, culture supernatants, or cell lysates were added into 384-well plates. Next, 25 µl of LgBiT protein or HiBiT peptide-containing Nano Glo HiBiT Lytic Detection Reagent (Promega) was added to the wells. After incubation at 37 °C for 1 h, luminescence signals were measured using a CentroXS3. For analysis of p24 concentration, HIV-1 p24 Antigen ELISA 2.0 (Zeptometrix, Buffalo, NY, USA) was used according to the manufacturer's instructions.

**Infection assay in a 96-well plate**

The target cells expressing CD4 and CCR5 were seeded in 96-well cell culture plates (2 × 10^4 cells/well). A day after seeding, cells were infected with a dose-adjusted pseudotyped virus with 10 µg/ml polybrene. A day after infection, the supernatant was removed and Steady-Glo Luciferase assay reagent (Promega) was added to dissolve cells to measure f-Luc activities.

**Statistical analysis**
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Statistically significant differences between the mean values were determined using a two-tailed Student’s t-test. All data are representative of at least two independent experiments. The values represent the means of triplicate samples ± SD. P values less than 0.05 were considered statistically significant. The P value for each analysis was indicated in each figure.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.
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Figure Legends

Figure 1. Conformational changes of gp41 subunit of HIV-1 Env during membrane fusion
(A) A schematic representation of the conformational changes of the gp41 subunit during membrane fusion. After interaction of gp120 with the receptor/coreceptor, CD4, and chemokine receptors (CKRs), gp41 undergoes dramatic conformational changes during membrane fusion. Pre- and post-fusion structures of gp41 are shown. For clarity, only one gp120/gp41 monomer is shown. The NHR and CHR portions of gp41 are shown in blue and yellow ribbons, respectively. In a prefusion structure, the gp120 subunit is shown in a surface-filled model. The post-fusion structure only depicts the gp41 subunit. The portion of gp41, which undergoes a dramatic loop-to-helix transition, is indicated by green color. The positions of glutamine residues (Q552 in NHR and Q653 in HXB2 CHR, respectively) are shown in red. The numbering of the residues is based on HXB2 Env (corresponding number in JRFL can be obtained by subtracting 9 from the numbering of HXB2; therefore, Q in JRFL Env will correspond to Q543 and Q644, respectively). The image was created by Chimera (UCSF) and was based on PDB 5FUU and 1ENV. (B) The structure and organization of a six-helix bundle (6HB). Side view and top view of a trimeric 6HB (PDB 1Env) are shown in the left and middle, respectively. The color coordination is the same as that in (A). The conserved glutamine residues are shown as in (A). The helical diagram of the portion of 6HB containing three NHRs (NHR1, 2, and 3) and one CHR (CHR1) is shown (right) to show the interacting residues in 6HB. The residues in a heptad repeat are assigned with a conventional (abcdefg) designation.

Figure 2. The primary structure of gp41 and mutants studied in this study. (A) The primary structures of HIV-1 Env of HXB2 and JRFL are shown (B2 stands for HXB2 and FL for JRFL). The upper sequence is for the region of NHR, and the bottom sequence is for CHR. The difference in the sequence between HXB2 and JRFL is indicated by the different amino acid residues of JRFL shown below the HXB2 sequence. The positions of α-helices based on the structural analyses are shown by colored boxes (pale blue and orange) above and below the sequences. The portions of 6HB depicted in Fig. 1 are shown by blue and yellow boxes between the NHR and CHR sequences. The positions of “a” and “d” in the heptad repeats are shown above the sequences. The arrowheads show the positions of the alanine insertion in α9. The mutant is named by the position of the inserted alanine; for example, in 641+A, the inserted alanine residue occupies position 641. (B) The amino acid sequences of the mutants used in this study are shown with a one letter code for amino acid residues. The positions of the inserted alanine residues are shown in red. The box indicates position 644 in the original sequence of JRFL Env.

Figure 3. Analyses of the cell-cell fusion activities of the alanine insertion mutants. (A), (B) The syncytia formation assay of the mutants. The expression vector for each mutant was transfected into CD4+CCR5+ cells, and the syncytia formation was evaluated by microscopy. A representative field for each mutant is shown (upper panel: phase contrast, lower panel: Hoechst stain; bar = 100 µm) in (A). The relative degree of syncytia formation was evaluated by counting the number of nuclei in the syncytia by the total number of nuclei in the observed fields. The wild type value was set as 1 (B). (C) The immunoblotting of the mutants. The expression and processing of gp160 were examined by immunoblotting using an anti-gp120 antibody (upper panel). The ratios of gp120/gp160 (processing of Env) or gp160 or gp120/GAPDH (expression level of Env) were determined by densitometry (lower panel). (D) The DSP assay to detect fusion pore formation between effector and target cells. The raw DSP values and normalized values by the surface level of Env are shown. **: statistically significant (P < 0.01). Error bars represent S.D. The representative results of two independent experiments are shown.
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Figure 4. Addition of a PH domain to LgBiT reduces the background signal of the R-BiT assay. (A) The target cells, 293 cells expressing CD4, CCR5, and Halo-LgBiT, were infected with Vpr-HiBiT-containing HIV-1 with pseudotyped JRFL Env in the absence (Cont) or presence of CCR5 inhibitor (maraviroc, MVC, 1 µg/ml) or with the antagonistic competitor of HiBiT, DrkBiT peptide (DrkBiT, 1 µg/ml). The reporter signal from re-constituted Nluc (Vpr-HiBiT and Halo-LgBiT) was monitored at 15, 30, and 60 min after infection. (B) The ratio between the signals obtained with the addition of DMSO or MVC presented in (A) is shown. (C) The FLuc activity expressed from the packaged fluc reporter gene using the same samples shown in (A) was measured at 24 h after infection with or without the FLuc substrate. (D) Effect of the engineering of the LgBiT reporter in the R-BiT assay on the level of background signals. Target 293 cells expressing CD4, CCR5, and various LgBiT derivatives were infected with Vpr-HiBiT-containing HIV-1 pseudotyped with JRFL Env in the presence (MVC) or absence (DMSO) of MVC. The Nluc-derived signals were measured. (E) The ratio between the signals obtained with (MVC) or without MVC (DMSO) in (D) is shown. (F) The FLuc activity, using the same samples shown in (D), was measured at 24 h after infection with or without the FLuc substrate. (G) The ratio between the Nluc signals for the cell supernatant and cell lysate was calculated. RLU: relative light unit. Error bars represent SD. The representative results of two independent experiments are shown.

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Figure 6. Analysis of the fusion activities of gp41 mutants in the virus-cell fusion assays. (A) The assay using the **new split-Nluc based reporter (R-BiT assay)**. The effect of JRFL gp41 mutation on viral infection was measured by the R-BiT assay. The target 293 cells expressing CD4, CCR5, and PH-Halo-LgBiT were used. In the R-BiT assay, the Nluc reporter signal from reassociated Vpr-HiBiT and PH-Halo-LgBiT was monitored at 15, 30, and 60 min after infection. (RLU: relative light unit. **: statistically significant \( P < 0.01 \)) (B) The result of the conventional FLuc-based fusion assay for the gp41 mutants is shown. The dose-adjusted pseudotyped viruses were used to infect target cells as described in the Materials and Methods section. The expression of the reporter gene (firefly luciferase) was measured after 48 h of infection. RLU: relative light unit. No virus control and VLP without Env were used as controls (negative and no env, respectively). Error bars represent S.D. RLU: relative light unit. **: statistically significant \( P < 0.01 \). (C) The analysis of the protein profiles of VLPs. The result of the immunoblotting using anti-gp120, anti-gp41, and anti-p24 antibodies as described in the Materials and Methods section is shown. The names of the corresponding viral proteins are shown on the right side. The positions of the bands of the molecular weight marker are indicated on the left side in kDa. (D) The result of the measurement of the band intensities; the ratios of the intensity of the bands to that of p24 band are determined, and the values for the wild type was set as 1. Error bars represent SD. The representative results of two independent experiments are shown.
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