Clearly different mechanisms of enhancement of short-lived Nef-mediated viral infectivity between SIV and HIV-1

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Abstract

Background: One of the major functions of Nef is in the enhancement of the infectivity of the human and simian immunodeficiency viruses (HIV and SIV, respectively). However, the detailed mechanism of the enhancement of viral infectivity by Nef remains unclear. Additionally, studies of mechanisms by which Nef enhances the infectivity of SIV are not as intensive as those of HIV-1.

Methods: We generated short-lived Nef constructed by fusing Nef to a proteasome-mediated protein degradation sequence to characterize the Nef role in viral infectivity.

Results: The apparent expression level of the short-lived Nef was found to be extremely lower than that of the wild-type Nef. Moreover, the expression level of the short-lived Nef increased with the treatment with a proteasome inhibitor. The infectivity of HIV-1 with the short-lived Nef was significantly lower than that with the wild-type Nef. On the other hand, the short-lived Nef enhanced the infectivity of SIV\textsubscript{mac239}, an ability observed to be interestingly equivalent to that of the wild-type Nef. The short-lived Nef was not detected in SIV\textsubscript{mac239}, but the wild-type Nef was, suggesting that the incorporation of Nef into SIV\textsubscript{mac239} is not important for the enhancement of SIV\textsubscript{mac239} infectivity.

Conclusions: Altogether, the findings suggest that the mechanisms of infectivity enhancement by Nef are different between HIV-1 and SIV\textsubscript{mac239}. Lastly, we propose the following hypothesis: even when the expression level of a protein is extremely low, the protein may still be sufficiently functional.

Keywords: SIV, HIV, Nef, Retrovirus infectivity

Background
The negative regulatory factor (Nef), a 27–35-kDa non-enzymatic protein, is an accessory protein of human and simian immunodeficiency viruses (HIV and SIV, respectively) that enhances viral replication; it is associated with the pathogenesis induced by these viruses both in vivo and in vitro [1-4]. Furthermore, Nef has many functional motifs for contact to host proteins [5], through which it can serve as a molecular adaptor, and exert multiple functions, like the downregulation of CD4 and major histocompatibility complex (MHC) class I [6]. N-Myristoylation occurs at the N-terminus of Nef [7], whose posttranslational modification is essential for its multiple functions [6].

One of the major functions of Nef is in the enhancement of viral infectivity, which is independent of CD4 [8] or MHC class I [9] downregulation. Many studies have suggested that the Nef incorporation into virions or the association of virions with Nef is responsible for the enhancement of viral infectivity mediated by Nef [10-15]. Welker et al. estimated that approximately 10 Nef molecules are incorporated into HIV-1 virions [13], much fewer than the 275 molecules of another accessory protein, Vpr [16], which also has the capability to enhance viral infectivity [17,18]. In many reports [15,19-25], it has been suggested that Nef incorporated into virions could exert its action in the early steps of infection, although the
molecular mechanism of this phenomenon remains substantially unclear [26]. Although Nef is clearly one of the factors for the pathogenesis induced by SIV in vivo [3], cell culture studies of the role of Nef in the enhancement of the infectivity of SIV [27] are not as intensive as those of HIV-1 described above. We, therefore, examined whether the Nef incorporation into virions or the association of virions with Nef is also important for the enhancement of the infectivity of SIVmac239 as well as of HIV-1.

In this study, we generated short-lived Nef constructed by fusing Nef to a proteasome-mediated protein degradation sequence, by which the balance between the synthesis and degradation of Nef can be changed. Consequently, the apparent expression level of Nef fused to the degradation sequence becomes much lower than that of the wild-type Nef. Accordingly, the frequency of the incorporation of the short-lived Nef into virions or the association of virions with the short-lived Nef would be lower than that with the wild-type Nef. Interestingly, the infectivity of SIVmac239 with the short-lived Nef was equivalent to that with the wild-type Nef. On the other hand, the infectivity of HIV-1NL4-3 with the short-lived Nef was significantly lower than that with the wild-type Nef. These results suggest that the mechanisms of the Nef-mediated enhancement of infectivity are different between SIV and HIV-1.

Results

Construction of short-lived Nef expression vector
Some degradation signals conferring instability on proteins have been found, which include the CL peptide [28] and a murine ornithine decarboxylase (MODC) PEST region [29]. These signals induce a rapid protein degradation mediated by a proteasome, in which the CL peptide requires ubiquitination prior to degradation, whereas the PEST sequence requires no ubiquitination [14]. The CL peptide and PEST sequence convert stable proteins into unstable ones by attachment as fusion proteins [28,30-32], whose apparent expression levels could be much lower than those of the original proteins [30-33]. In this study, we utilized a combination of two protein degradation sequences of the CL peptide and PEST sequence, namely, the CP sequence, to generate short-lived Nef. We hypothesized that the apparent expression level of short-lived Nef is lower than that of wild-type Nef, which we evaluated to determine whether the Nef incorporation into virions is associated with the enhancement of the infectivity of SIVmac239.

To detect Nef by western immunoblot analysis under the same conditions, the V5 epitope was appended at the C-terminus of Nef to preserve the N-myristoylation site of Nef, which is an essential posttranslational modification for Nef functions [6,7]. The CP sequence was fused at the C-terminus of V5-tagged Nef (Figure 1A).

Both wild-type (WT) Nef and CP-fused Nef were expressed under the control of the cytomegalovirus (CMV) promoter. To verify the effect of the fused CP sequence on Nef expression level, HEK293 cells were transfected with pNefmac239-WT or pNefmac239-CP. After a 48 h cultivation, HEK293 cells transfected with pNefmac239-CP were treated with 20 μM MG132 for 0 or 6 h. The cells were collected and subjected to SDS-PAGE and western immunoblot analysis to detect Nefmac239-WT and Nefmac239-CP using the anti-V5 antibody. β-Actin was also detected using the anti-β-actin antibody, as described in Materials and Methods (B). The relative band intensities of Nefmac239-CP and β-actin were quantified using Fujifilm Image Gauge software. The expression levels of Nefmac239-CP relative to those of β-actin treated with and without MG132 were calculated using the obtained intensities and compared (C).

Figure 1 Construction of short-lived Nef expression vector.
Schematic representations of Nef-WT and Nef-CP used in this study (A). HEK293 cells were transfected with pNefmac239-WT or pNefmac239-CP. After a 48 h cultivation, HEK293 cells transfected with pNefmac239-CP were treated with 20 μM MG132 for 0 or 6 h. The cells were collected and subjected to SDS-PAGE and western immunoblot analysis to detect Nefmac239-WT and Nefmac239-CP using the anti-V5 antibody. β-Actin was also detected using the anti-β-actin antibody, as described in Methods (B). The relative band intensities of Nefmac239-CP and β-actin were quantified using Fujifilm Image Gauge software. The expression levels of Nefmac239-CP relative to those of β-actin treated with and without MG132 were calculated using the obtained intensities and compared (C).
proteasome inhibitor MG132 for 6 h before the cell lysis was carried out, and Nef\textsubscript{mac239-CP} was then detected by western immunoblot analysis. As shown in the two lanes on the right of Figure 1B, the apparent expression level of Nef\textsubscript{mac239-CP} increased time-dependently about 6-fold in the cells treated with MG132 for 6 h relative to the level in the untreated cells (Figure 1C). This result validates that the relatively low expression level of Nef\textsubscript{mac239-CP} is due to its short-life property induced by fusing Nef to the CP sequence.

**Figure 2** Difference in incorporation level in virions between Nef\textsubscript{mac239-WT} and Nef\textsubscript{mac239-CP}.

Next, we examined the incorporation level of Nef\textsubscript{mac239-CP} in the virions of SIV\textsubscript{mac239}. The nef-defective SIV\textsubscript{mac239} provirus with the reporter gene was cotransfected into HEK293 cells with or without the plasmid encoding Nef\textsubscript{mac239-WT} or Nef\textsubscript{mac239-CP}. After a 72 h cultivation, the supernatants were collected by ultracentrifugation and used in the viral preparation, as described in Materials and Methods. The viruses were lysed and subjected to immunoblot analysis to detect Nef. Uniform virion loading was confirmed by western immunoblot analysis of the SIV p27 core antigen. As shown in the top panel of Figure 2, Nef\textsubscript{mac239-WT} was detected in SIV\textsubscript{mac239}. However, under the same conditions, Nef\textsubscript{mac239-CP} was not detected in SIV\textsubscript{mac239}, as in the mock transfection control. This result clearly indicates that the incorporation level of Nef\textsubscript{mac239-WT} is higher than that of Nef\textsubscript{mac239-CP}.

HIV-1 Nef enhances the incorporation of the envelope protein into virions [34]. Accordingly, we simultaneously examined the incorporation level of the envelope protein SIV\textsubscript{mac239} virions. Western immunoblot analysis of the viral envelope protein was performed. As shown in the middle panel of Figure 2, the incorporation level of the envelope protein in SIV\textsubscript{mac239} virions with Nef\textsubscript{mac239-CP}, which was equivalent to that of the mock transfection control, was clearly lower than that with Nef\textsubscript{mac239-WT}. Altogether, the phenotype of SIV\textsubscript{mac239} virions with Nef\textsubscript{mac239-CP} was similar to that without Nef\textsubscript{mac239}.

**Figure 3A** Comparison of effects of Nef-WTs and Nef-CPs on enhancement of SIV\textsubscript{mac239} infectivity.

The viral infectivity enhanced by Nef\textsubscript{mac239-CP} was compared with that enhanced by Nef\textsubscript{mac239-WT} in SIV\textsubscript{mac239}. To examine the effects of Nef\textsubscript{mac239-WT} and Nef\textsubscript{mac239-CP} on SIV infectivity, HEK293 cells were cotransfected with a nef-defective SIV\textsubscript{mac239} provirus with the luciferase reporter gene and the plasmid expressing Nef\textsubscript{mac239-WT} or Nef\textsubscript{mac239-CP}. Additionally, plasmids expressing nonmyristoylated G2A mutants, i.e., Nef\textsubscript{mac239-G2A} and Nef\textsubscript{mac239-CP-G2A}, were also used as nonfunctional Nef. The expression level of each Nef in the virus-producing cells was examined by western immunoblot analysis of the obtained cell lysate. Indicator cells, i.e., MAGIC-5 cells, were inoculated with each supernatant containing SIV\textsubscript{mac239}, and the luciferase activity of the MAGIC-5 cells was measured after a 48 h cultivation, as described in Materials and Methods. The MAGIC-5 cell line is a HeLa cell derivative modified to express CD4 (the primary receptor) and CCR5 (the coreceptor), respectively, for the infection by SIV\textsubscript{mac239} and CCR5-tropic HIV-1. The infectivity of each virus was compensated for by the amount of each SIV p27 antigen. The expression level of each Nef\textsubscript{mac239} derivative in each type of producer cell is shown in the top panel of Figure 3A. Uniform sample loading was confirmed by western immunoblot analysis of actin. The expression levels of Nef\textsubscript{mac239-CP} and Nef\textsubscript{mac239-CP-G2A} were much lower than those of Nef\textsubscript{mac239-WT} and Nef\textsubscript{mac239-G2A}.

The comparison of the relative infectivities of SIV\textsubscript{mac239} produced from HEK293 cells expressing all the Nef\textsubscript{mac239} derivatives is shown in the bottom panel of Figure 3A. The enhancement of the viral infectivity by Nef\textsubscript{mac239-WT}
Figure 3 (See legend on next page.)
was observed as expected whereas the enhancement of the viral infectivity by Nef\textsubscript{mac239}\textsubscript{-}G2A, a nonmyristoylated and nonfunctional mutant, was not observed. Under this condition, SIV\textsubscript{mac239} with Nef\textsubscript{mac239}–WT was about 4-fold more infectious than the virus without Nef. Unexpectedly, the enhancement of the viral infectivity by Nef\textsubscript{mac239}\textsubscript{-}CP was observed, although the apparent expression level of Nef\textsubscript{mac239}\textsubscript{-}CP was much lower than that of Nef\textsubscript{mac239}–WT. Interestingly, no significant difference in the enhancement of viral infectivity between Nef\textsubscript{mac239}\textsubscript{-}WT and Nef\textsubscript{mac239}\textsubscript{-}CP was observed. The enhancement by Nef\textsubscript{mac239}\textsubscript{-}CP was abolished by the nonmyristoylated G2A mutation, as in the case of the G2A mutation of Nef\textsubscript{mac239}–WT.

It was examined whether Nef from the HIV-1 strain can enhance SIV\textsubscript{mac239} infectivity similarly to Nef\textsubscript{mac239}\textsubscript{-}CP. Nef from the JR-CSF strain of HIV-1, namely, Nef\textsubscript{JR-CSF} was chosen in this study because the apparent expression level of Nef\textsubscript{JR-CSF}\textsubscript{-}WT is reported to be relatively higher than those of other HIV-1 strains. The top panel of Figure 3B shows the expressions of Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP in the lysate of the virus-producing cells determined by western immunoblot analysis. The apparent expression level of Nef\textsubscript{JR-CSF}\textsubscript{-}CP generated by fusion of the Nef to the CP sequence was much lower than that of Nef\textsubscript{JR-CSF}–WT, and the mobility of the weakly expressed Nef\textsubscript{JR-CSF}\textsubscript{-}CP was lower than that of Nef\textsubscript{JR-CSF}–WT as in the case of Nef\textsubscript{mac239}\textsubscript{-}CP (Figure 1B). Under this condition of the virus-producing cells, the infectivity of each produced SIV\textsubscript{mac239} with or without Nef\textsubscript{JR-CSF}–WT or Nef\textsubscript{JR-CSF}\textsubscript{-}CP was examined. As shown in the bottom panel of Figure 3B, significant enhancement of the viral infectivity by Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP in comparison with that in the case of a virus without Nef was observed. Moreover, no significant difference in the enhancement of viral infectivity between Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP was observed, as in the case of Nef\textsubscript{mac239}\textsubscript{-}WT and Nef\textsubscript{mac239}\textsubscript{-}CP. Altogether, these results indicate that Nef-WTs and Nef-CPs show equivalent activities for the enhancement of SIV\textsubscript{mac239} infectivity in the case of Nef from not only SIV\textsubscript{mac239} but also HIV-1, even though the apparent expression levels were markedly different between Nef-WTs and Nef-CPs.

Comparison of effects of Nef-WTs and Nef-CPs on enhancement of HIV-1 infectivity

Many studies have suggested that the Nef incorporation into virions or the association of virions with Nef is responsible for the enhancement of the infectivity of HIV-1 [10-13,15]. Accordingly, it has been expected that the activity of Nef-CPs would be lower than that of Nef-WTs in HIV-1. The effects of Nef-WTs and Nef-CPs on HIV-1 infectivity were evaluated as follows. HEK293 cells were cotransfected with an env- and nef-defective HIV-1–NL4-3 provirus with the luciferase reporter gene inserted into nef, a plasmid encoding a CCR5-tropic JR-FL envelope protein, and a plasmid encoding either Nef\textsubscript{JR-CSF}–WT, Nef\textsubscript{JR-CSF}\textsubscript{-}CP, Nef\textsubscript{mac239}\textsubscript{-}WT, or Nef\textsubscript{mac239}\textsubscript{-}CP. To examine the infectivity, MAGIC-5 cells were infected with the supernatants including CCR5-tropic HIV-1–NL4-3 and incubated for 48 h. Then, luciferase activity as the indicator of infectivity was measured. The infectivity of each virus was compensated for by the amount of the p24 antigen of each virus. The top panel of Figure 3C shows the expressions of Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP in the virus-producing HEK293 cells determined by western immunoblot analysis. Uniform sample loading was confirmed by western immunoblot analysis of actin. The expression level of Nef\textsubscript{JR-CSF}\textsubscript{-}CP was much lower than that of Nef\textsubscript{JR-CSF}–WT, whose expression profile was the same as that of Nef\textsubscript{mac239}\textsubscript{-}WT or Nef\textsubscript{mac239}\textsubscript{-}CP expressed in SIV\textsubscript{mac239}–WT (Figure 3B). Under this condition of the virus-producing cells, the infectivity of each produced SIV\textsubscript{mac239} with or without Nef\textsubscript{ JR-CSF}–WT or Nef\textsubscript{JR-CSF}\textsubscript{-}CP was examined. As shown in the bottom panel of Figure 3B, significant enhancement of the viral infectivity by Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP in comparison with that in the case of a virus without Nef was observed. Moreover, no significant difference in the enhancement of viral infectivity between Nef\textsubscript{JR-CSF}–WT and Nef\textsubscript{JR-CSF}\textsubscript{-}CP was observed, as in the case of Nef\textsubscript{mac239}\textsubscript{-}WT and Nef\textsubscript{mac239}\textsubscript{-}CP. Altogether, these results indicate that Nef-WTs and Nef-CPs show equivalent activities for the enhancement of SIV\textsubscript{mac239} infectivity in the case of Nef from not only SIV\textsubscript{mac239} but also HIV-1, even though the apparent expression levels were markedly different between Nef-WTs and Nef-CPs.
lower than that of Nefmac239-WT, showing the same expression profile as that of each Nefmac239 derivative expressed in the SIVmac239-producing cells (Figure 3A). Under this condition of the virus-producing cells, the infectivity of HIV-1NL4-3 with Nefmac239-WT was significantly much higher than that of HIV-1NL4-3 without Nef. On the other hand, the infectivity of HIV-1NL4-3 with Nefmac239-CP was significantly lower than that of HIV-1NL4-3 with Nefmac239-WT, which correlated with the expression levels of Nefmac239-WT and Nefmac239-CP.

Taken together, the above results indicate that the activity of enhancement of HIV-1 infectivity by Nef-CPs is significantly lower than that by Nef-WTs, which were from both SIVmac239 and HIV-1JR-CSF. The difference in this activity between Nef-CPs and Nef-WTs was considered to be associated with the difference in apparent expression level between Nef-CPs and Nef-WTs. These results for HIV-1 could support previous reports suggesting that the Nef incorporation into virions or the association of virions with Nef is responsible for the enhancement of the infectivity of HIV-1 [10,13,15]. Therefore, the substantial enhancement of infectivity of SIVmac239 by Nef-CP is again considered to be a unique property.

Low expression level of Nefmac239 is not sufficient for the enhancement of SIVmac239 infectivity

It was indicated that Nef-CPs could enhance the infectivity of SIVmac239, whose apparent activity was equivalent to those of Nef-WTs, although the apparent expression levels of Nef-CPs were much lower than those of Nef-WTs (Figures 3A and B). Typically, the activities of Nef-CPs are lower than those of Nef-WTs for the enhancement of SIVmac239 infectivity, similarly to HIV-1 (Figures 3C and D). However, the profiles of the activities of Nef-WTs and Nef-CPs for SIVmac239 were unexpectedly but interestingly different from those for HIV-1. Here, we hypothesized that even a very low expression level of Nef is sufficient to enhance the infectivity of SIVmac239. To test this hypothesis, a plasmid DNA encoding for Nefmac239-CP was used. As shown in Figure 4A, a graded decrease in the expression level of Nefmac239-WT was observed in a Nefmac239-WT encoding DNA dose-dependent manner. A very low expression level of Nefmac239-CP from 1 μg of DNA was observed, which was much lower than that of Nefmac239-WT from 1 μg of DNA and was almost equivalent to that of Nefmac239-WT from 0.05 μg of DNA. Under this condition of each virus-producing cell type, the infectivity of each SIVmac239 produced is shown in Figure 4B. A graded decrease in infectivity of the virus was observed in a transfected Nefmac239-WT encoding DNA dose-dependent manner. The infectivity of SIVmac239 produced from the cells transfected with 0.05 μg of DNA encoding Nefmac239-CP was significantly lower than that transfected with 1 μg of DNA encoding Nefmac239-CP. On the other hand, the infectivity of SIVmac239 produced by cells transfected with 1 μg of DNA encoding Nefmac239-CP was almost similar to that transfected with 1 μg of DNA encoding Nefmac239-CP, whose result was the same as that shown in Figure 3A. Taken together, these results indicate that such a low expression level of Nefmac239-WT, which is similar to that of Nefmac239-CP, is not sufficient to enhance the infectivity of SIVmac239.

Discussion

Although the enhancement of viral infectivity by Nef is clear, the molecular mechanism by which Nef enhances the infectivity of HIV-1 and SIV remains unclear. In this study, it was clearly indicated that there is a significant difference between SIVmac239 and HIV-1NL4-3 in their response to the infectivity enhancement by Nef-CP, suggesting that the molecular mechanisms of Nef action differ between SIVmac239 and HIV-1NL4-3.

It has been reported that HIV-1 Nef enhances the incorporation of envelope proteins into virions, which is associated with the increase in HIV-1 infectivity [34]. Additionally, Nef incorporated into virions is suggested to directly enhance virion infectivity in the early steps of infection by HIV-1 [24,25]. In this study, it was observed that the amount of envelope protein of SIVmac239 without Nefmac239-WT was clearly lower than that with Nefmac239-WT, suggesting that Nefmac239 can also enhance the incorporation of envelope proteins into SIVmac239 as in the case of HIV-1 [34]. Thus, the amount of envelope protein of SIVmac239 with Nefmac239-CP was clearly lower than that with Nefmac239-WT, which was similar to that without Nefmac239-WT (Figure 2). On the other hand, the infectivity of SIVmac239 with Nefmac239-CP was interestingly equivalent to that with Nefmac239-WT (Figure 3A). Accordingly, it is suggested that the enhancement of the incorporation of the envelope protein into SIVmac239 by
Nef is not associated with the enhancement of viral infectivity by Nef. Additionally, the direct action of Nef incorporated to virions in the early step of SIV infection is suggested to be not essential for the enhancement of viral infectivity. Taken together, the results suggest that the molecular mechanisms of the Nef-mediated enhancement of viral infectivity are different between HIV-1 and SIV. Furthermore, a mechanism by which Nef can enhance SIV infectivity inside virus-producing cells, which is not associated with the incorporation of envelope proteins into the virions, may exist.

The N-myristoylation of Nef is essential for the major functions of Nef [6]. The lack of effect of nonmyristoylated Nef-CP-G2A on the enhancement of viral infectivity (Figure 3A) indicates that the function of Nef-CP is also dependent on N-myristoylation and may be dependent on membrane localization. Since it was also confirmed that such a low expression level of Nef-WT from 0.05 μg of DNA, similar to that
of Nef<sub>mac239</sub>-CP from 1 μg of DNA, was not sufficient to enhance the infectivity of SIV<sub>mac239</sub> (Figure 4), it is hypothesized that a protein synthesis process for producing a quorum of Nef<sub>mac239</sub> molecules within a short time in SIV<sub>mac239</sub>-producing cells is required for the enhancement of viral infectivity even if the duration of the presence of each molecule of Nef<sub>mac239</sub> is extremely short in the cell.

Lastly, we propose the following hypothesis: even when the expression level of a protein such as Nef-CP is extremely low, the protein may still be sufficiently functional; this novel and unique hypothesis might be greatly applicable in various fields such as biochemistry and cell biology. Thus, a rapid turnover by not only rapid protein degradation but also efficient production of a protein through a strong promoter activity in transcription or efficient translation might be essential for the protein to function sufficiently.

**Conclusion**

The findings suggest that the mechanisms of the enhancement of viral infectivity by Nef are different between HIV-1 and SIV<sub>mac239</sub>. Additionally, we propose the following hypothesis: even when the expression level of a protein is extremely low, the protein may still be sufficiently functional.

**Methods**

**Materials**

The infectious HIV-1 NL4-3 expression vectors pNL-CH [35] and psvJR-FLenv [36] were kindly gifted by Dr. Ron Swanstrom of the UNC Center for AIDS Research, University of North Carolina at Chapel Hill, Chapel Hill, NC. The infectious HIV-1 expression vector pYK-JR-CSF was obtained from the NIH AIDS reagent program.

**Constructions of nef- and env-deficient and reporter-gene-introduced SIVmac239 and HIV-1 expression vectors**

Firefly-luciferase-encoding DNA was amplified by PCR using a pGL4.14 [luc2/Hygro] vector (Promega, Madison, WI) and inserted into the nef-coding region of pBRmac239, coding for SIV<sub>mac239</sub> proviral DNA, as previously reported [37]. The nef-deficient and luciferase-gene-inserted SIV<sub>mac239</sub> proviral DNA vector was named pBRmac239Δnef<sub>luc</sub>. To delete env of pNL-CH, the Stel and BsaBI sites in env were digested by the corresponding enzymes and the linear DNA obtained was subjected to a ligation reaction, whose vector encoding the env-deficient proviral DNA was named pNL-CHΔenv. Firefly-luciferase-encoding DNA was amplified in PCR using a pGL4.14 [luc2/Hygro] vector and inserted into the nef-coding region using the XhoI site of pNL-CHΔenv, whose vector with the luciferase reporter gene and without env and nef was named pNL-CHΔenvΔnef<sub>luc</sub>. The infectious HIV-1 expression vector pYK-JR-CSF was obtained from the NIH AIDS reagent program.

**Nef expression vectors**

Nef-coding DNAs were amplified by PCR using the corresponding proviral DNA templates (pBRmac239 for Nef<sub>mac239</sub> and pYK-JR-CSF for Nef<sub>JR-CSF</sub>) and subcloned into pcDNA3.1D/V5-His TOPO according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). A Gly<sup>2</sup>-to-Ala<sup>2</sup> (G2A) mutation in nef was introduced using site-directed mutagenesis. To introduce the short-life property into the expressed Nef proteins, DNA encoding a sequence of the CL peptide and the PEST, namely, the CP sequence, was amplified from pGL4.78 [hRlucCP] Hygro (Promega, Madison, WI). The DNA was then linked after the region of the V5 epitope tag encoding DNA using the AgeI site of each Nef expression vector. All the constructed Nef expression vectors were named as follows: pNef<sub>mac239</sub>-WT, pNef<sub>mac239</sub>-CP, pNef<sub>mac239</sub>-G2A, pNef<sub>mac239</sub>-CP-G2A, pNef<sub>JR-CSF</sub>-WT, and pNef<sub>JR-CSF</sub>-CP.

**Viral preparation**

For the preparation of SIV<sub>mac239</sub>, pBRmac239Δnef<sub>luc</sub> was transiently cotransfected with either pNef<sub>mac239</sub>-WT, pNef<sub>mac239</sub>-CP, pNef<sub>mac239</sub>-G2A, pNef<sub>mac239</sub>-CP-G2A, pNef<sub>JR-CSF</sub>-WT, pNef<sub>JR-CSF</sub>-CP, or an empty vector into HEK293 cells using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA). For the preparation of CCR5 tropic HIV-1NL4-3, pNL-CHΔenvΔnef<sub>luc</sub> and psvJR-FLenv were transiently cotransfected with either pNef<sub>mac239</sub>-WT, pNef<sub>mac239</sub>-CP, pNef<sub>JR-CSF</sub>-WT, pNef<sub>JR-CSF</sub>-CP, or an empty vector into HEK293 cells. At 72 h posttransfection, the supernatants were collected and filtered through a 0.45 μm filter and subjected to ELISA for p27 or p24 antigen or viral infectivity assay. For virion preparation, the supernatants were subjected to ultracentrifugation as previously described [38].

**Quantification of SIV p27 and HIV-1 p24 antigen in supernatant**

Each cell-free supernatant was filtered using a 0.45-μm-pore-size filter and subjected to ELISA for SIV p27 or HIV p24 (ZeptoMetrix Corporation, Buffalo, NY), according to the manufacturer’s instructions.

**Measurement of viral infectivity**

MAGIC-5 cells were inoculated with the supernatant as previously described [39]. After a 72 h cultivation, the cells were washed with PBS(−) and lysed with Cell Culture Lysis Reagent (Promega, Madison, WI). The luminescence of the firefly luciferase reporter was measured with a luciferase assay system (Promega, Madison, WI) using a Wallac ARVO™ SX 1420 luminometer (Perkin-Elmer, Walthman, MA).
Cell lysis and western immunoblot analysis

The cells were washed, lysed, and subjected to 5–20% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The antibodies used in different immunoblottings were as follows: anti-V5 antibody (Invitrogen, Carlsbad, CA), anti-actin antibody (Oncogene, San Diego, CA), anti-gp130 SIV monoclonal antibody (Immuno Diagnostics, Inc., Woburn, MA), and anti-SIVmac239 monkey serum. Immuno-complexes were detected using appropriate peroxidase-conjugated secondary antibodies, followed by visualization by chemiluminescence detection (NEL Life Science Products, Boston, MA, USA) with LAS4000 (GE Healthcare, Buckingham, England). The intensities of the bands were quantified with FujiFilm Image Gauge Software.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

KH performed most of the experiments. NT designed the experiments and participated in the experiments. NT and KH wrote the manuscript. SS and SM supervised the project. All authors are read and approved the final manuscript.

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