Functional association between the *nef* gene product and *gag-pol* region of HIV-1

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Abstract Nef gene function is diverse among virus isolates of primate immunodeficiency viruses. We found differential effects of *nef* mutation on the virus replication between two HIV-1 clones, NL432 and LAI. The *nef* mutation in NL432 affected the infectivity more severely compared with that in LAI, although the Nef functions of both clones were comparable. Analysis with a series of chimeric viruses between NL432 and LAI revealed that the *gag-pol* region was responsible for the differential effect of *nef* mutation. The functional association between Nef and *gag-pol* suggested that one of the potential targets of Nef was located within the *gag-pol* region.

Key words: Human immunodeficiency virus type 1; Nef; Gag; Pol

1. Introduction

In addition to the prototypic retroviral *gag*, *pol*, and *env* genes, lentiviruses encode a number of accessory genes that are unique to primate immunodeficiency viruses. The *nef* gene is one of the accessory genes and its function is essential for the maintenance of high levels of viral replication and pathogenesis in adult macaques infected with simian immunodeficiency virus [1]. The contribution of Nef to viral replication and pathogenesis has been highlighted by recent findings showing that human immunodeficiency virus (HIV) isolates from some long-term non-progressors have deletions in the *nef* gene [2].

Unlike the in vivo results, the function of Nef in vitro had been unclear. The in vitro analysis is perplexed by the fact that the effect of Nef on viral infectivity is varied among the isolates and is dependent upon cell type [3,4]. However, evidence is accumulating that Nef enhances viral infectivity in vitro. The upregulation of viral replication by Nef has been reported to be mediated through an increase in proviral DNA synthesis [5,6], but the mechanism remains unknown for the most part. Among the known activities of Nef, its association with cellular kinases is likely to be involved in the enhancement of viral infectivity. Nef has been reported to interfere with the function of the Src family kinases, Lck and Hck, through the interaction between a Src homology 3 domain of each kinase and a highly conserved polyproline type II helix-structured proline motif within Nef, the disruption of which affects HIV replication [7,8].

The diversity of Nef function among HIV-1 isolates might reflect the different pathogenicity of the virus in vivo. It is, therefore, worth analyzing the mechanism(s) for the diversity to clarify the molecular function of Nef. In this study, we found diverse effects of *nef* mutation on viral replication in two HIV-1 clones, NL432 and LAI, and show a functional association between Nef and the *gag-pol* region.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells and SW480 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 10% heat-inactivated fetal bovine serum (FBS). The $CD4^+$ human leukemia cell line A3.01 [9] was maintained in RPMI 1640 medium containing 10% FBS. HeLa-CD4 cells were maintained in D-MEM supplemented with 10% FBS, and 0.2 mg/ml Geneticin (G418; Gibco/BRL). HeLa-CD4-LTR-β-gal cells (MAGI cells; multinuclear activation of a galactosidase indicator) [10] were propagated in D-MEM supplemented with 10% FBS, 0.2 mg/ml Geneticin, and 0.1 mg/ml hygromycin B (Sigma). For transfection, the calcium phosphate co-precipitation [11] and the modified DEAE-dextran methods [12] were used for adherent and suspension cells, respectively.

2.2. Provirus DNA constructs

An infectious proviral clone of HIV-1, pNL432 (GenBank accession number G328415) and its *nef* mutant, pNL432-Δ*nef*, have been described (previously designated pNL-Xh) [13]. The pLAI *nef* mutant clone, pLAI-Δ*nef*, was constructed from pLAI (GenBank accession number K02013, kindly provided by Dr. Keith Peden) by inserting the stop codon linker into the *Xho*I site found in the *nef* open reading frame. The chimeric virus clones, pLain DNAs, were constructed by a standard DNA manipulation (Figs. 1A and 3). The pLain-Δ*rev*-Luc series shown in Fig. 3 were constructed from pLain series which have a point mutation at the *rev* ATG initiation codon (ATG is substituted with ATC) and an insertion of the luciferase coding gene (derived from pGL3-basic, Clontech) in the *nef* region (*Xho*I site).

2.3. Analysis of virus growth kinetics

The viral stocks used for the infection were prepared from HeLa cells transfected with infectious molecular clones. A3.01 cells (1×10^6 cells) were infected for 6 h with the virus stock in 1 ml of growth medium in the presence of 2 μg/ml polybrene. The amounts of input virus were adjusted according to the amount of p24 (30 ng of p24, which was determined by HIV-1 p24 antigen ELISA; Cellular Products). Fresh growth medium (4 ml) was added to the infected cells after the incubation. Every 2 days after infection, the culture supernatant was harvested and the RT activity was monitored by a standard method [14]. The RT activity was determined as the photo-stimulated luminescence (PSL) with a BAS-2000 image analyzer (Fuji Film).

2.4. MAGI assay

The viral stocks were obtained from the HeLa or SW480 cells transfected with DNA clones. The viruses from infected A3.01 cells were

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Abbreviations: HIV-1, human immunodeficiency virus type 1; MAGI, multinuclear activation of a galactosidase indicator; RT, reverse transcriptase; WT, wild type; GFP, green fluorescent protein

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also used as viral stock. MAGI cells were infected with the same amount of virus (adjusted with the RT activity) in the presence of 2 μg/ml polybrene, and the β-galactosidase activity in the cells was monitored at 20 h after infection by the Luminescent β-galactosidase Genetic Reporter System II (Clontech).

2.5. Expression vectors

The Nef-GFP fusion genes were obtained by inserting the *nef* genes of NL432 and LAI into pEGFP-1 plasmid (Clontech), then the fusion genes were subcloned into the $pCMV_4$ mammalian expression vector [15]. The obtained expression plasmids were designated pNL-Nef-GFP and pLAI-Nef-GFP. The *rev* expression plasmid, pCMV-F-Rev, was obtained by inserting the *rev* gene into $pCMV_4$. The *rev* gene was derived from the rev expression plasmid, pCV-1, which has been described previously [16].

2.6. Single-round infection

pLain-Δ*rev*-Luc, pCMV-F-Rev, and pNL-Nef-GFP were used for single-round infection assay. pLain-Δ*rev*-Luc was transfected into HeLa cells, and Rev and Nef-GFP proteins were supplied from the expression plasmids in *trans*. CD4-HeLa cells were infected with an equal amount of virus. Three days after infection, the luciferase activity in the cells was assayed with the Luciferase Assay System (Promega).

2.7. Assays of viral entry

Viral stocks were obtained as the supernatants of HeLa cells transfected with DNA clones. MAGI cells (1×10^6) were incubated with the viruses for 4 h at 37°C in the presence of 2 μg/ml polybrene. At 4 h after infection, the cells were washed, trypsinized, and resuspended in FBS. One part containing 1/10 of the total number of cells was lysed in phosphate-buffered saline containing 1% Triton X-100 and analyzed by p24 antigen ELISA to quantify intracellular CA protein [17]. The other portion was placed back in culture medium at 37°C and cultured for an additional 14 h, then subjected to MAGI assay.

3. Results

3.1. Differential effect of nef mutation on the infectivity of NL432 and LAI

The effect of *nef* mutation on the virus replication was reported to be dependent on the virus strain [3]. We compared the growth kinetics of wild type (WT) and *nef* mutant viruses derived from NL432 and LAI in A3.01 cells (Fig. 1B). These two viruses are T-cell line tropic and closely related to each other. In the case of the NL432 clone, the growth kinetics of *nef* mutant (Δ*nef*) was apparently retarded in comparison with that of WT. However, the *nef* mutation in LAI had only a slight effect on virus replication, indicating that the requirement of Nef function for the virus replication was different between these HIV-1 clones. A similar result was obtained with another T-cell line, H9 (data not shown).

3.2. Nef function is comparable between NL432 and LAI viruses

The amino acid homology between Nefs of NL432 and LAI is 98.1% as calculated by analyzing software (GENETIX-

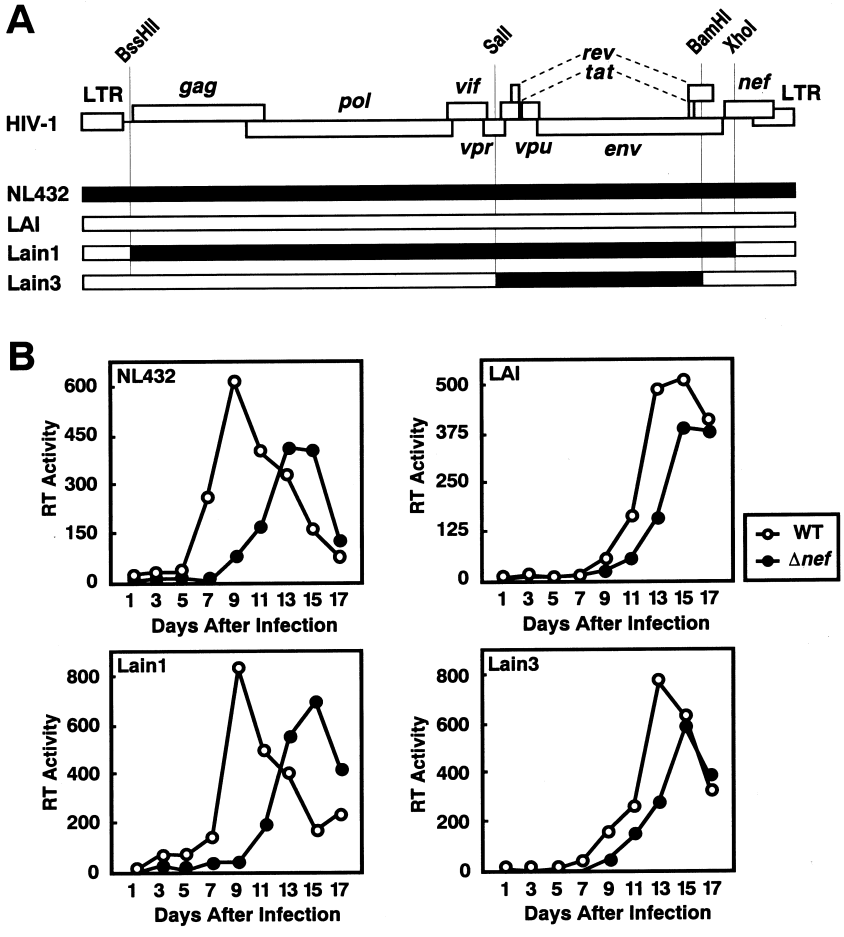

Fig. 1. Functional diversity of Nef function between NL432 and LAI viruses. A: Genomic structures of NL432, LAI, and the chimeric viruses, Lain1 and Lain3. B: Growth kinetics of WT and Δ*nef* viruses. A3.01 cells were infected with NL432, LAI, Lain1 or Lain3. On the day after infection indicated in the figure, the RT activity in the culture supernatant was monitored. The RT activity is indicated as PSL units.

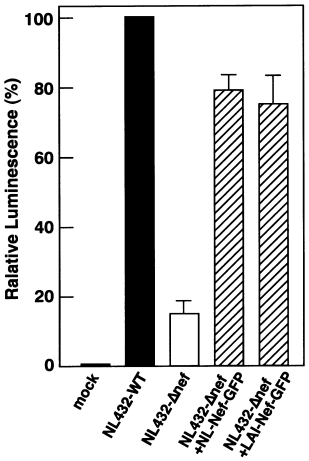

Fig. 2. Functional assay of the Nef activity on enhancement of viral infectivity. Viruses were produced from HeLa cells by cotransfection of pNL432-Δ*nef* and Nef expression plasmid (pNL-Nef-GFP or pLAI-Nef-GFP), and their infectivities were assayed using MAGI cells as described in Fig. 1. The result represents the average of three independent experiments, and the standard deviations are indicated as error bars. The β-galactosidase activities in the cells infected with WT viruses (NL432) and in the mock-infected cells (mock) are indicated as 100% and 0%, respectively.

Mac, Software Development Co., Ltd.), which indicates that these Nefs are closely related to each other. It was possible, however, that the differential effect of the *nef* mutation was determined by the diversity of the *nef* gene. A comparison of amino acid sequences revealed four substitutions between these Nefs, which are located outside the functional domains reported previously [1]. To test whether or not these substitutions conferred the difference of Nef function, the infectivity of *nef*-deficient NL432 virus (NL432-Δnef) complemented with Nef in *trans* was analyzed using MAGI reporter cells (Fig. 2). Nefs derived from NL432 and LAI were expressed as GFP fusion proteins. The Nef-GFP protein in the transfected cells was easily detectable and primarily localized in plasma membrane as reported previously (data not shown) [18]. The mutation of the *nef* gene reduced the infectivity of NL432 severely (about 15% infectivity of WT). In the complementation experiment, NL-Nef-GFP and LAI-Nef-GFP enhanced the infectivity of NL432-Δ*nef* to the same extent, indicating that there was no functional difference between these Nefs as to the enhancement effect on infectivity. Similar results were obtained with the viral stocks derived from either SW480 or A3.01 cells. The infectivities of Nef complemented viruses were lower than that of WT virus, suggesting the expression level of Nef was not optimal for the enhancement of viral infectivity. It was also possible that the effect of multiround infection augmented the difference, because Nef complementation had an effect only on the first round of infection.

3.3. Detailed mapping of the determinant for the Nef mutational effect

The result described above indicated that the different effect

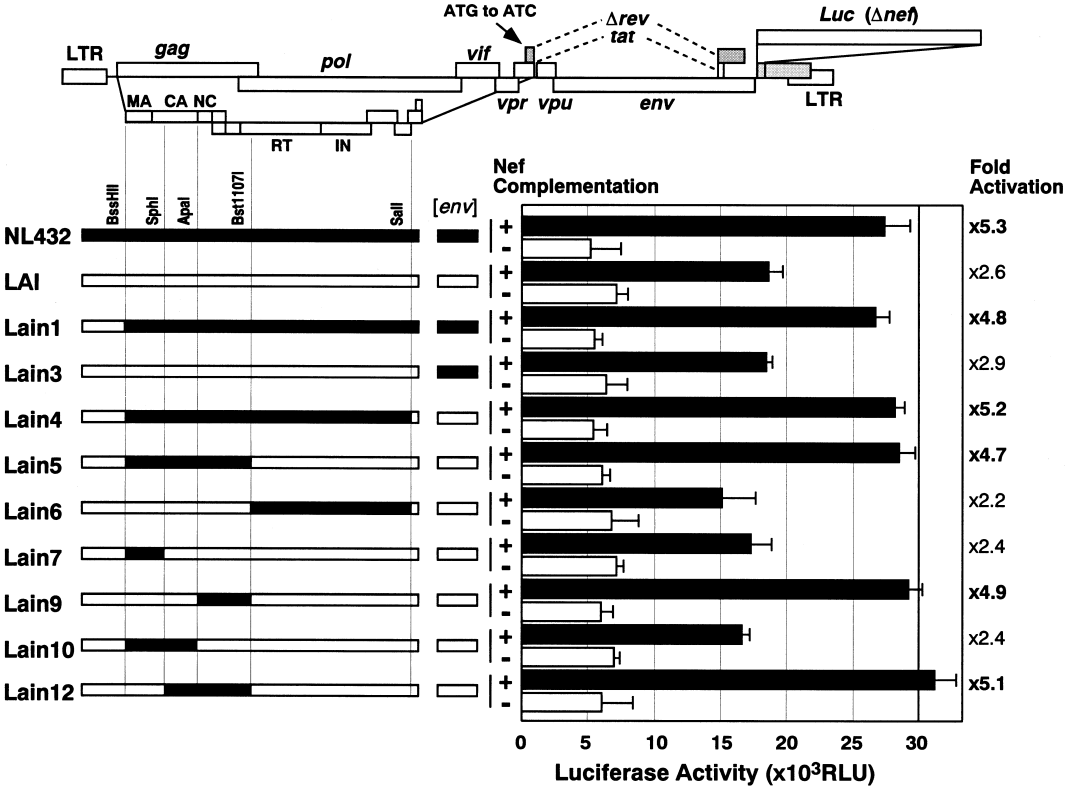

Fig. 3. Detailed mapping of the determinant for the Nef mutational effect by a single-round assay. Genomic organizations of chimeric viruses are depicted in the figure (closed and open bars indicate the sequences derived from NL432 and LAI, respectively). The origin of *env* is indicated separately. pLain-Δ*rev*-Luc and pCMV-F-Rev and/or pNL-Nef-GFP were co-transfected into HeLa cells and the obtained viruses were used for single-round infection assay. CD4-HeLa cells were infected with the same amount of virus (adjusted with RT activity), and the luciferase activity in the cells was monitored at 40 h after infection by a standard assay. Data were obtained from five independent experiments, and the standard deviations are indicated as error bars. The luciferase activities are shown as relative luciferase activity (RLU) and the fold activation of virion infectivity by *nef* is indicated.

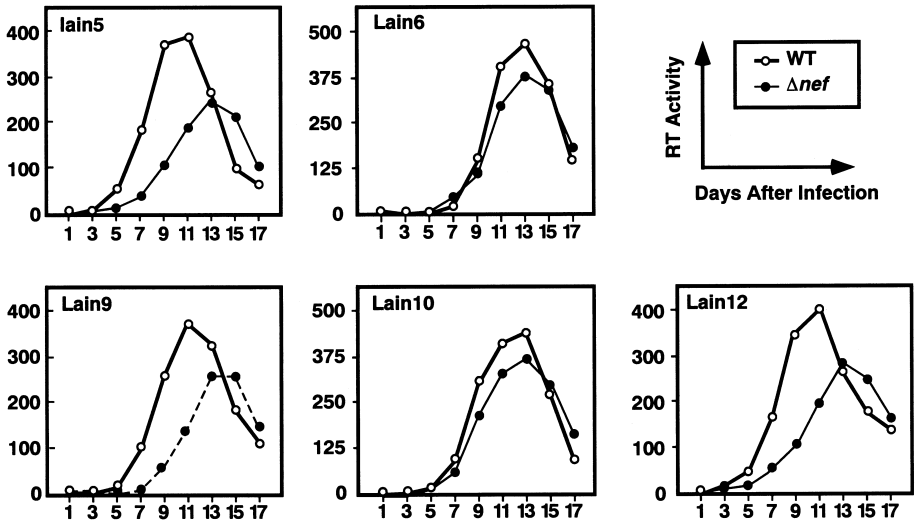

Fig. 4. Growth capabilities of the chimeric viruses. Replication-competent Lain viruses which are similar to those shown in Fig. 3 but have intact *rev* were used for the infection assay. The virus stocks used for the infection were prepared from HeLa cells transfected with plasmid DNA clones. The RT activity post infection was monitored by a standard assay, which is indicated as PSL units.

of *nef* mutation in NL432 and LAI was determined by region(s) other than the *nef* gene. To identify the determinant, two chimeric clones (Lain1 and Lain3) were constructed and their growth abilities in A3.01 were analyzed. The 3′ portions of Lain1 and Lain3 were both derived from NL432 (Fig. 1A; *Sal*I-*Bam*HI). As shown in Fig. 1B, Lain1 and Lain3 exhibited NL432-type and LAI-type phenotypes for *nef* mutation, respectively, suggesting the determinant for the different effect of *nef* mutation was located in the 5′ region (*Bss*HII-*Sal*I) encompassing the *gag-pol*, *vif* and 5′ portion of *vpr*.

To map the determinant(s) for the *nef* mutation in detail, additional chimeric viruses (Lain series) were constructed as depicted in Fig. 3. The effect of *nef* mutation in these chimeric viruses was monitored by a modified single-round infection assay. Virus stocks were obtained from the cells transfected with pLain-Δ*rev*-Luc, pCMV-F-Rev and pNL-Nef-GFP. The virus produced from the transfected cells contained a mutation in *rev*; thus it was defective for virion production, making it possible to analyze the progress of the early phase of infection by monitoring the luciferase activity without the effect of multi-round infection. The result clearly indicated that the *nef* mutation affected the early phase of infection and that the *Apa*I-*Bst*1107I portion in the *gag-pol* region conferred the differential effect of *nef* mutation observed between NL432 and LAI. By comparing the infectivities of Lain-Δ*rev*-Luc viruses, it was revealed that the infectivities of *nef*-deficient viruses were almost the same but those having the NL432-derived sequence encompassing *Apa*I-*Bst*1107I (NL432 and Lain1, 4, 5, 9, and 12) were much enhanced by Nef complementation, suggesting the augmentation of viral infectivity by Nef was determined by this region.

We also analyzed the growth kinetics of replication-competent Lain viruses in A3.01 cells and some of the results are shown in Fig. 4. The minimal region for the NL-like phenotype for *nef* mutation could be mapped within the *Apa*I-*Bst*1107I in *gag-pol* region, supporting the result obtained with single-round infection assay.

Table 1

	Relative RT activity[a] (10^3)	Entry[b] (ng/10^6 cells)	Infectivity[c]	
			RLU	Fold activation[d]
Mock	0	0	0	–
NL432	12.09	10.0	261 000	×7.4
NL432-Δ*nef*	10.77	11.2	35 326	
LAI	12.88	9.7	169 965	×3.8
LAI-Δ*nef*	11.33	10.1	44 765	
Lain3	15.47	10.5	170 329	×4.6
Lain3-Δ*nef*	13.37	10.3	37 343	
Lain5	16.17	8.3	272 131	×8.4
Lain5-Δ*nef*	16.64	9.1	32 417	
Lain9	13.36	8.5	260 878	×7.1
Lain9-Δ*nef*	14.05	9.0	36 522	
Lain10	15.98	7.6	159 699	×4.5
Lain10-Δ*nef*	15.79	8.0	35 454	

[a]RT activity (PSL unit) of culture supernatant of the transfected HeLa cells.
[b]Virus entry was quantified at 4 h after infection by measuring the intracellular HIV-1 p24 protein concentration of the MAGI cells infected with equal amount of viral stock. The results represent nanograms of p24 antigen per 10^6 cells.
[c]Viral infectivity was measured by the luminescent MAGI assay and the results are shown as relative luminescent units (RLU).
[d]Enhancement of viral infectivity by Nef function is indicated.

3.4. Effect of nef mutation after virion entry in the early phase of the infectious cycle

There was no significant difference in virus production from the transfected cells between WT and *nef*-deficient Lain viruses (some of the results are shown in Table 1: Relative RT activity), and the results described above indicated that Nef modified the viral infectivity in the early phase of infection. To clarify which step in the early phase was regulated by the *gag-pol* region and Nef, we examined the virion attachment/penetration with Lain viruses following a standard method for virion entry assay [17]. MAGI cells were infected with the same amount of Lain viruses and the amount of virus entering the cells was estimated by p24 ELISA (Table 1: Entry). Similar concentrations of intracellular p24 protein were detected for both the WT and *nef* mutant viruses, indicating that the impaired progression of the early infection phase by *nef* mutation was not due to a block at the point of virus entry. Analysis of proviral DNA synthesis of early (R-U5) and late (R-gag) products was also performed 18 h after infection (data not shown). It appeared that the efficiency of DNA synthesis was decreased with the *nef*-defective viruses as reported previously, but we could not detect significant effects on the DNA synthesis by the substitution of the *gag-pol* gene, even though the PCR analysis was performed in a quantitative range. These results indicated the effects by the *gag-pol* region and Nef took place after virion entry in the early phase of infection.

We also performed a MAGI assay in a parallel experiment (Table 1: Infectivity). As mentioned above, the Nef effect in augmentation of viral infectivity was determined by the *gag-pol* region (*Apa*I-*Bst*1107I), suggesting the possibility that one of the functional targets of Nef is located within the *gag-pol* region.

4. Discussion

The function of Nef has been reported to be dependent upon cell type and virus strain but little is known about the molecular mechanism of the dependence. In this report, we described the diversity of the mutational effect of the *nef* gene between NL432 and LAI viruses. The aims of our study were to obtain a clue as to the functional diversity of Nef among HIV-1 strains and to elucidate the molecular mechanism of Nef function in the enhancement of viral infectivity. Nef is reported to have multi-functions in the viral life cycle, including the enhancement of virion infectivity and the downregulation of CD4 and MHC class I molecule expressions [19,20], therefore, it is possible that several pathways are responsible for the diversity of Nef function among HIV-1 strains. We expected that it would be possible to limit the determinants for the diversity by using closely related HIV-1 clones, NL432 and LAI. The 3′ halves of these viruses are almost the same but there are several substitutions in both nucleotide and amino acid sequences. Even in their 5′ portion, the homologies of Gag and Pol are 93.2% and 96.5%, respectively, indicating these HIV-1 clones are closely related.

There are many reports describing that the *nef* mutation reduces viral infectivity in vitro. Although we observed the Nefs of both NL432 and LAI could stimulate viral infectivity, it was more marked with NL432. Although these two clones are closely related, there were four amino acid substitutions between these Nefs. We examined the functional diversity of Nefs of both viruses in the enhancement of virion infectivity by MAGI assay, and no functional difference was observed between these Nefs (Fig. 2), suggesting that the determinant(s) was located in another part of the viral genome.

To identify the determinant(s) of the diversity, a series of chimeric viruses were constructed and their infectivities were analyzed. The results indicated that the *Apa*I-*Bst*1107I region within the *gag-pol* gene was responsible for the diversity, which could encode portions of Gag (NC, p6) and Pol (PR, N-terminal portion of RT). A contribution of either PR or RT to the diversity seemed unlikely because the *nef* mutation had no effect on the virion production measured with RT activity. PR mutation is reported to have no effect on virion production but the RT activity of PR-deficient virions is greatly reduced. The mechanism by which the *gag* region regulates the viral infectivity in connection with Nef remains unclear. In the *gag* portion of the *Apa*I-*Bst*1107I region, there are six amino acid substitutions between NL432 and LAI, and 12 amino acids are inserted in the NC protein of LAI. One possible explanation is that the *gag* region acts as a target of Nef in enhancing infectivity. As shown in Fig. 3 and Table 1, augmentation of viral infectivity by Nef appeared to be determined by the *gag-pol* region, suggesting that Nef modified the viral infectivity through interaction with the Gag-Pol precursor. Although there is no report describing the direct interaction between the Gag-Pol precursor and Nef, these proteins and several cellular kinases were reported to be co-localized in the cytoplasmic membrane [21,22], suggesting that Gag-Pol and Nef form a complex via cellular kinases. It was reported that Nef enhanced the phosphorylation of MA through Ser/Thr kinase, resulting in the enhancement of viral infectivity [23]. This report supports the idea that Gag-Pol is one of the targets of Nef. Although the MA portion was not related to the diversity described in this report, it is possible that other regions within Gag-Pol also serve as a target for Nef-associated kinase. It is worth investigating the association between Nef and the kinases that regulate the infectivity through the phosphorylation of virion components.

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