Conservation of the central proline-rich (PxxP) motifs of human immunodeficiency virus type 1 Nef protein during the disease progression in two hemophiliac patients

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Abstract **The *nef* gene is considered to play a crucial role in the development of acquired immunodeficiency syndrome (AIDS). In this study, we analyzed the sequence of *nef* quasispecies obtained from replication-competent HIV-1 isolates from two Japanese hemophiliac patients infected with HIV-1. At least 10 *nef* clones were isolated at each time point and a total of 75 individual *nef* quasispecies were sequenced. We observed a gradual increase in genetic diversity of the *nef* gene over time. Among the various functional regions of Nef protein, myristoylation site and the central PXXP (SH3 ligand) motifs were well conserved. The scattered regions responsible for downregulation of CD4 and class I MHC were also conserved. These data suggest that these functions of Nef may be involved throughout the disease process.**

Key words: HIV-1; *nef*; AIDS; SH3 ligand; Molecular evolution

1. Introduction

Nef is a myristoylated 27–30 kDa protein encoded by primate lentiviruses and is produced in abundance at an early stage of the viral life cycle (reviewed in [1]). Nef is considered to be an important determinant of acquired immunodeficiency syndrome (AIDS) pathogenesis [2–5]. For example, Nef was shown to be necessary for maintaining high viral loads and inducing AIDS-like disease in rhesus monkeys infected with SIVmac239 [2]. Moreover, molecular studies with human long-term non-progressors of human immunodeficiency virus type 1 (HIV-1) infection indicated that the intact *nef* gene sequence was required for progression of AIDS [3–5].

A number of reports have demonstrated that various biological effects of Nef were mediated through interaction with various cellular factors. It has been demonstrated that the Src homology region 3 (SH3) ligand region (PXXP) of Nef protein is involved in the association with Src-family proteins (Src, Lyn, Hck, Lck and Fyn) and affects signal transduction pathways such as T-cell activation [6–11]. The N-terminal myristoylation site and an acidic region have been implicated in the viral infectivity in which Nef appears to facilitate reverse transcription in the incoming virion pre-integration complex [12–16]. Nef has also been shown to downregulate cell-surface CD4 protein by facilitating the endocytotic pathway through its interaction with cellular factors such a catalytic subunit of the vacuolar ATPase, NBP-1 [17–22].

In the present study, we have isolated the replication competent HIV-1 isolates over time from two Japanese hemophiliacs patients infected with HIV-1 and analyzed the temporal profile of molecular evolution of the *nef* gene in vivo. We observed that the complexity of *nef* sequence heterogeneity was increased during the progression of HIV-1 infection. We also found that some functional domains of Nef were conserved in both patients.

2. Materials and methods

2.1. Patients material

Patients A and B are hemophiliacs who had been infected with HIV-1 through a contaminated batch of factor VIII. The CD4 cell counts, the CD4/CD8 ratio and the disease status of patients A and B at each time of sample procurement are shown in Table 1. During the periods when samples were isolated, patient A was treated with ddI from May 1991 to January 1992 and 5′-azido-3′-deoxythymidine (AZT) therapy from August 1992. Patient B was treated with ddI and AZT therapy from April 1993.

2.2. Virus isolation

Virus isolation was performed according to the method previously described [23,24]. Briefly, peripheral blood mononuclear cells (PBMCs) were Ficoll-separated from the heparinized blood and cocultivated with phytohaemagglutinin (PHA)-stimulated PBMCs from HIV-1-negative healthy donors in RPMI 1640 medium containing 20% fetal calf serum, 0.25 U/ml recombinant interleukin-2 (Takeda Chemical Industries, Osaka, Japan) and 50 U/ml of anti-human interferon-α antibody (JCR Pharmaceuticals, Kobe, Japan). These infected cells were maintained in culture for only a short period (3 to 4 days) before the cellular genomic DNA was separated. Virus stocks were grown only once in PHA-stimulated PBMC cultures.

2.3. Polymerase chain reaction (PCR) amplification of the nef gene

In order to purify the cellular genomic DNA, the HIV-1-infected PBMCs were resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K), incubated at 50°C for 18 h, extracted by phenol-chloroform, and then ethanol-precipitated according to the standard method. The purified DNA from PBMC cultures infected with each HIV-1 isolate was served as the template for PCR. The HIV-1 *nef* region was amplified by using Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. PCR primer pairs were designed in order to cover most of the heterologous *nef* gene populations according to the HIV-1 sequences registered in GenBank. Among the four different primer sets we examined for *nef* gene amplification, the following two primer sets gave good amplification of the *nef* gene in most of the samples. The primer sequences we chose were as follows: primer pair 1: 5′nef1(5′-ATAG-

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Abbreviations: PCR, polymerase chain reaction; HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; SH3, Src homology region 3; PHA, phytohaemagglutinin; PBMC, peripheral blood mononuclear cells

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CAGTAGCTGAGGGGACAGATAG-3′) and 3′nef1 (5′-CTGGTC-TAACCAGAGCCCAGTACAGGCA-3′); primer pair 2: 5′nef2 (5′-CACATACCTAGAAGAATAAGACAGGGCT-3′) and 3′nef1.

2.4. Cloning and sequencing

PCR mixture was run on 1.2% low melting temperature agarose (FMC BioProducts, ME, USA) and the 847 bp DNA fragment (using the primer set 1) and the 812 bp DNA fragment (using the primer set 2) were recovered using GENECLEAN kit (BIO 101, CA, USA). The purified DNA was subsequently ligated into a PCR-blunt vector using the Zero blunt PCR cloning kit (Invitrogen, CA, USA). For each virus isolate at each time point, at least 10 individual plasmid clones containing the *nef* gene were sequenced using the Bigdye terminator cycle sequencing reaction kit (Perkin Elmer, CA, USA) and ABI PRISM 310 Genetic Analyzer (Perkin Elmer). Standard M13 universal and reverse primers as well as internal *nef*-specific primers (5′nef3, 5′-GGTTTTCCAGTCAGACCT-3′; 3′nef2, 5′-CTCTCCTTCATT-GGCCT-3′) were used as sequencing primers. The nucleotide sequence data in this paper had been submitted to the DDBJ, EMBL and GenBank databases and were assigned to the accession numbers: AB012809–AB012820, AB012821–AB012837, AB012839–AB012842 and AB026222–AB026247.

2.5. Analysis of the sequence data

Phylogenetic analyses were performed to investigate the similarity among the isolates in this study and previously reported HIV-1 strains, HIV-1SF2 [25] and HIV-1 LAI [26]. Using the computer program ODEN version 1.1.1 [27], the number of nucleotide substitutions per site and the genetic distances for all these isolates were estimated by the six-parameter method [28,29]. Based on these values, phylogenetic trees were constructed by the neighbor-joining method [30]. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times [31]. Sequence diversities (%) between pairs of DNA and amino acid sequences were calculated using DNADIST program and PROTDIST program, respectively, of the PHYLIP 3.5c program package [32].

3. Results and discussion

3.1. Sequence analysis of the Nef protein derived from patients A and B

Table 1 shows the patient profile at the time when the HIV-1 *nef* gene was obtained. At each time for each patient we isolated and sequenced at least 10 individual clones. From patient A, we obtained a total of 45 sequences. Among these clones, there were 37 distinct *nef* DNA clones. Since some of the clones shared amino acid sequences, although differed for their DNA sequences, a total of 34 distinct Nef amino acid sequences were obtained. From patient B, we obtained a total of 26 distinct *nef* DNA sequences and 23 distinct Nef amino acid sequences.

The aligned protein sequence data sets obtained from the replication competent HIV-1 isolates are demonstrated in Fig. 1. Amino acid sequence variation was found scattered throughout Nef protein and even from the visual inspection it was noted that the amino acid sequence heterogeneity became greater over time in each patient. Potential functional protein domains indicated by previous studies [19,20,33–36] are indicated in the top of this Fig. 1 (the details will be discussed in the later part of this report).

In each patient, there were some clones with the same amino acid sequence (for example with patient A, A-I-1, A-I-2 and A-I-3 had the same amino acid sequence although their DNA sequences were different; there were some other clones with the same amino acid sequence although they were isolated in different time points such as A-I-4, A-I-5 and A-II-6). The identical clones, containing the same DNA sequence, were obtained (such as A-I-6 (2 identical clones) and A-II-1 (4 identical clones)). However, no identical clones were found among the different time points. In patient B, six clones shared the same amino acid sequence (B-1-1(3), 2, 3 and 4), among which three clones shared even the same DNA sequence (B-I-1(3)). In both patients, duplication of 12 to 27 nucleotide were found in a restricted region known as 'variable region' (in clones A-II-2, A-II-3, A-II-4, A-II-5, A-IV-3, A-IV-4, A-IV-5, B-III-5(2) and B-III-8) [28]. Frame-shift mutants were noted in some clones (7 out of 45 clones in patient A and 6 out of 25 clones in patient B) and a mutant with premature termination was noted in one clone (B-I-8, patient B) as previously reported [3,4,33]. It was noted that B-I-5, in which additional nucleotide 'T' was inserted at the nucleotide position 227 of B-I-1(3) and thus resulted in an extensive frame-shift mutation.

3.2. Genetic diversity of nef gene over time during the disease progression

The nucleotide sequence diversities of the *nef* gene in both patients were evaluated using DNADIST program. As demonstrated in Fig. 2, the *nef* quasispecies increased in complexity over time. The temporal increase of complexity in patient A might be due to the emergence of other HIV-1 quasispecies with duplication at the 'variable region' in the 5′ region (clones A-II-2, A-II-3, A-II-4 and A-II-5) and that with +1 frameshift at nucleotide position 317 (clones A-II-1 and A-II-7) (Fig. 1). During the disease progression the genetic diversity of each *nef* quasispecies reached the average divergences of 7.33% and 7.34% at the latest stage of HIV-1 infection for patients A and B, respectively (Fig. 2). It was noted that the sequence diversity was rising even before the clinical criteria for diagnosis of AIDS was fulfilled.

To further examine the overall variability of the *nef* DNA sequence, phylogenetic trees were constructed using ODEN software (version 1.1.1) [27,29]. As demonstrated in Fig. 3, *nef* sequences obtained from the late clinical stages of patients (marked by open boxes) do not form distinct clusters, which is consistent with the results shown in Fig. 2. In contrast, *nef* sequences obtained from earlier stages form multiple distinct clusters (for example, A-I, A-II, and A-III quasispecies are separately grouped in patient A) as previously indicated by Plikat et al [35]. In both patients, the nucleotide sequence diversity within the same quasispecies obtained from the earlier stages of the disease had a limited heterogeneity, whereas that from the later stage (A-IV and B-III quasispecies obtained at the CDC classification stage 4) showed more extensive heterogeneity. The extents of genetic diversity of the *nef* gene observed in this study were similar to that obtained in the previous reports for *nef* [33] and *env* [37,38] genes by others.

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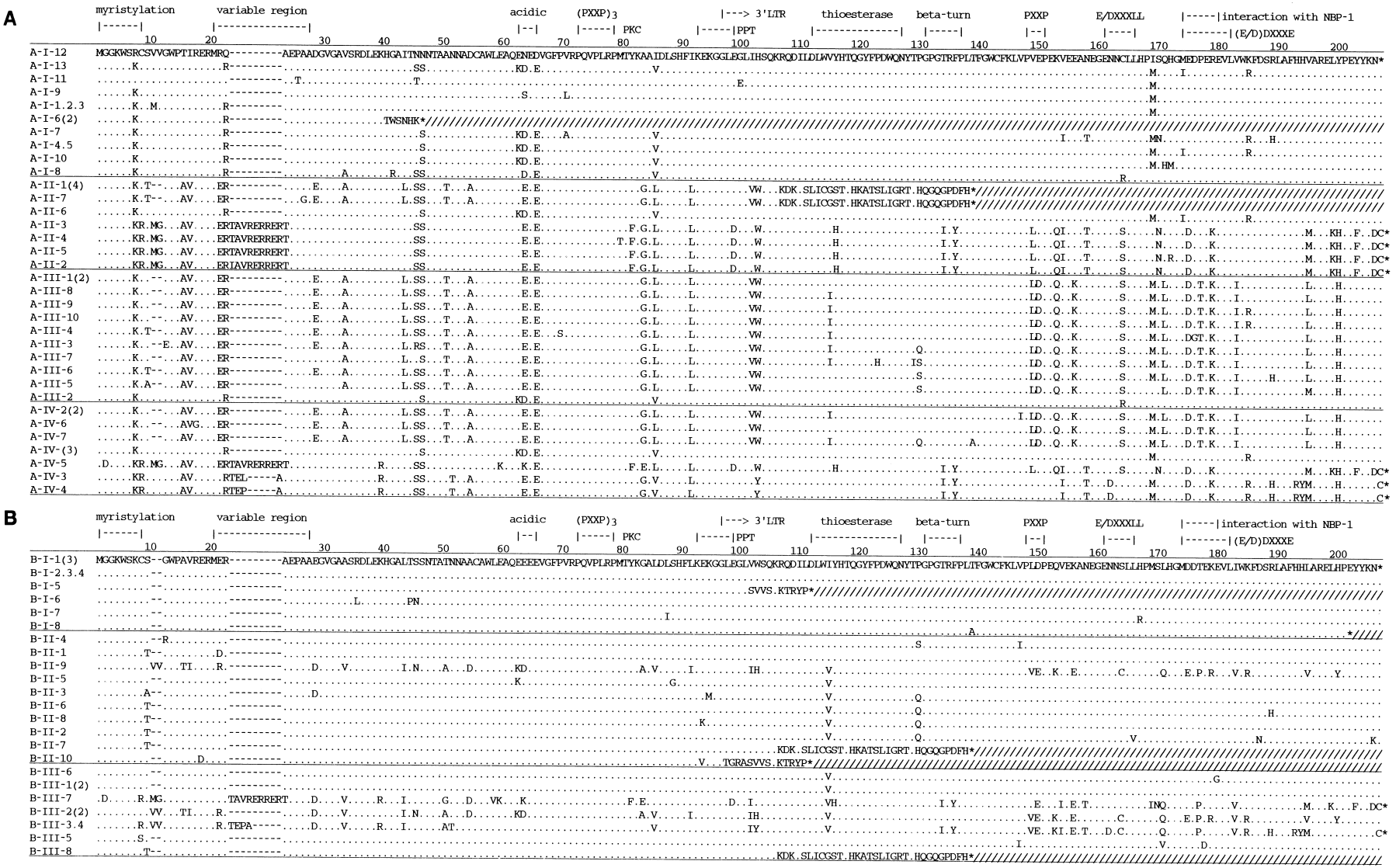
Fig. 1. Nef amino acid sequences corresponding to HIV-1 *nef* clones obtained from patients A and B. Amino acid sequences are aligned with clones A-I-12 and B-I-1 as standards. Gaps (-) are introduced to maximize homology. Asterisks (*), position of premature termination. Slashes (/), deletion. Dots (.), identical amino acids. LTR, long terminal repeat. PPT, polypurine tract.


A
myristylation
variable region
acidic
(PXXP)3
PKC
3'LTR
PPT
thioesterase
beta-turn
PXXP
E/DXXXLL
interaction with NBP-1
(E/D)DXXXE
A-I-12
A-I-13
A-I-11
A-I-9
A-I-1.2.3
A-I-6(2)
A-I-7
A-I-4.5
A-I-10
A-I-8
A-II-1(4)
A-II-7
A-II-6
A-II-3
A-II-4
A-II-5
A-II-2
A-III-1(2)
A-III-8
A-III-9
A-III-10
A-III-4
A-III-3
A-III-7
A-III-6
A-III-5
A-III-2
A-IV-2(2)
A-IV-6
A-IV-7
A-IV-(3)
A-IV-5
A-IV-3
A-IV-4
B
myristylation
variable region
acidic
(PXXP)3
PKC
3'LTR
PPT
thioesterase
beta-turn
PXXP
E/DXXXLL
interaction with NBP-1
(E/D)DXXXE
B-I-1(3)
B-I-2.3.4
B-I-5
B-I-6
B-I-7
B-I-8
B-II-4
B-II-1
B-II-9
B-II-5
B-II-3
B-II-6
B-II-8
B-II-2
B-II-7
B-II-10
B-III-6
B-III-1(2)
B-III-7
B-III-2(2)
B-III-3.4
B-III-5
B-III-8

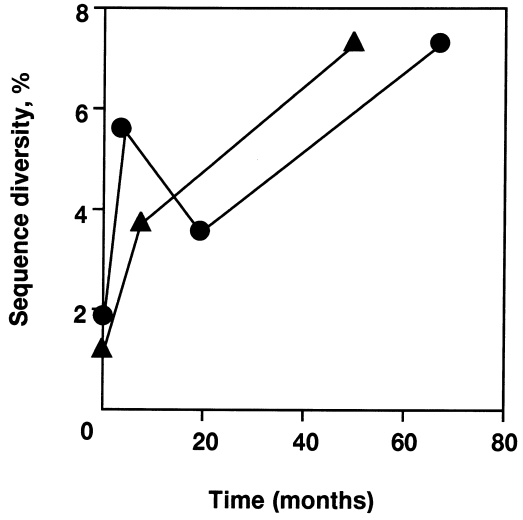


Fig. 2. Temporal increase of sequence diversity in the *nef* gene. Sequence diversity was calculated based on the distance for each pair of clones using the DNADIST program. Average diversity of patient A (●) and B (▲) at each time of virus isolation was indicated. The abscissa indicates the time scale (in months) with the time of first virus isolation as a starting point.

3.3. Conservation of functional Nef domains during disease progression

To examine whether positive selection is operating on the certain regions of *nef* gene in vivo, we evaluated the domain-specific sequence diversity both at the DNA and the protein levels (Fig. 4). Among various functional Nef domains defined by others, we found that the N-terminal myristoylation site and the SH3-binding motif $(PxxP)_3$ were well conserved in both patients (Fig. 1, 4). It was noted that genetic diversities of these regions at the protein level were much lower than those at the DNA level, especially for $(PXXP)_3$ which showed complete amino acid sequence conservation in patient B. We also found that in these regions most of the substitutions were synonymous in spite of the fact that there was increasing sequence diversity at the DNA level over time (Fig. 4). The potential thioesterase binding region (between 111 and 126 amino acids) was also conserved, although to a lesser extent [36]. These data suggest that the conserved Nef regions, thus their relevant biological functions, may be required for maintaining the virus to be competent for replication throughout the course of infection in vivo and that these functions may be required for the viral pathogenesis [2–5]. In spite of extensive investigations of the *nef* sequence heterogeneity, selective conservation of these regions during the disease progression in vivo has not been previously noted [33–35,39]. Since in these studies the *nef* gene was amplified directly from the patient plasma or the infected cells, it is likely that these *nef* sequences did not represent the replication-competent virus [40–43]. Although these defective viruses may kill the infected cells, they do not produce progeny virions and thus do not contribute to the disease progression.

Myristoylation of Nef protein at its N-terminus has been

Fig. 3. Phylogenetic tree of the HIV-1 *nef* DNA sequences. The relationships among 45 clones of patient A (A) and 30 clones of patient B (B) are demonstrated. The tree was constructed by the neighbor-joining method using a software ODEN version 1.1.1 [27]. One thousand bootstrap replications were performed. Bootstrap probabilities for major clusters are shown by percentages (%). Clones obtained from the late clinical stage (CDC classification 4) of these patients are identified by open boxes.

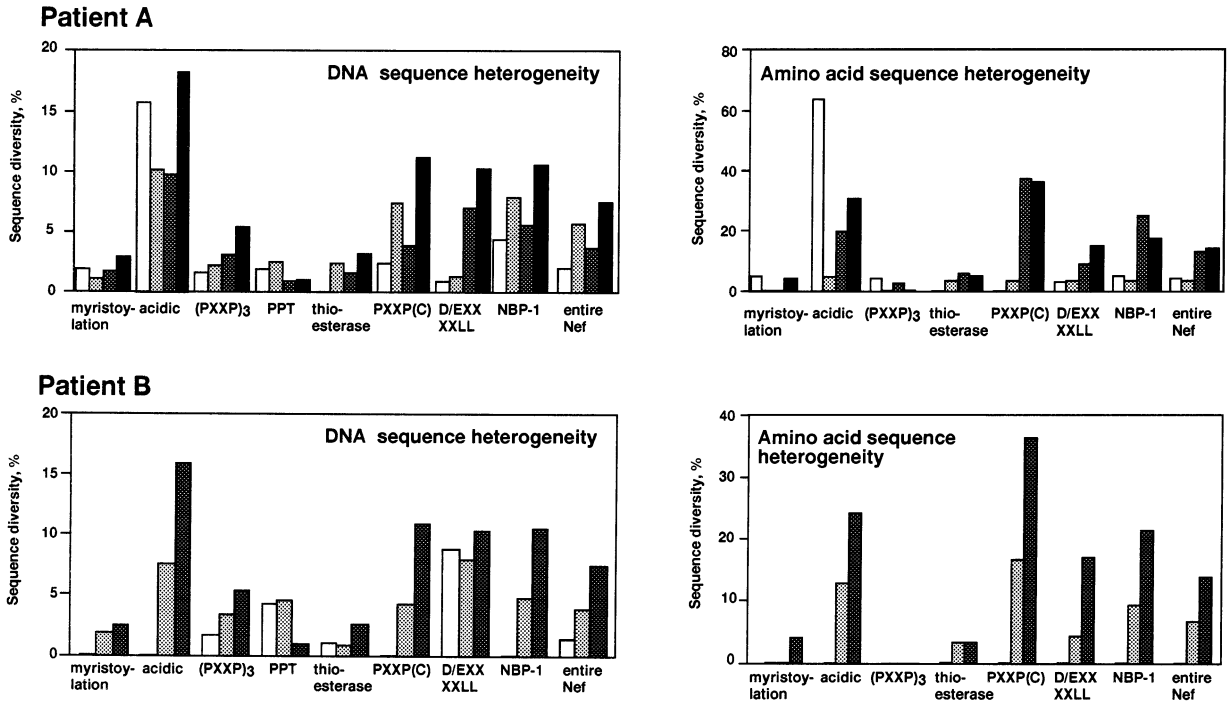


Fig. 4. DNA and amino acid sequence heterogeneity among the HIV-1 *nef* clones obtained from the serial samples of two Japanese AIDS patients (cases A and B). Functional domains indicated are according to the previous studies [19,20,33–35]. Sequence diversities were calculated by DNADIST and PROTDIST program of the PHYLIP 3.5c program package [29]. Calculation was performed with the *nef* quasispecies at each time of virus isolation in each patient. Patient A: (□) clones obtained in 1989.1, (lightly dotted square) 1989.5, (darkly dotted square) 1990.8. (■) 1994.8. Patient B: (□) clones obtained in 1990.7, (lightly dotted square) 1991.2, (darkly dotted square) 1994.9. See the text for the details of comparison.

implicated in its cytoplasmic membrane association [44] and in the viral pathogenicity [45]. Requirement of the N-terminal myristoylation, thus its membrane localization, has been indicated in the Nef-induced CD4 and class I MHC down regulation [12,13], T-cell signaling [14], and viral infectivity [15]. More importantly, Aldrovandi et al. [45] have recently demonstrated that the sole aberration of the myristoylation site resulted in the great loss of viral pathogenicity in vivo using SCID-hu mouse model in which human thymic tissue was implanted in SCID mice.

Nef has been shown to bind to SH3 domains of a subset of Src family tyrosine kinases [6–8] and a cellular serine/threonine kinase related to a p21 kinase (PAK), designated Nef-associated kinase [9,10] through the highly conserved central SH3 ligand motifs $(PXXP)_3$. Although the pathophysiological relevance of these protein-protein interactions remains to be established, it was suggested that Nef might interfere with T-cell signaling through its interaction with these kinase molecules. It is possible that juxtaposing these signal-transducing kinases that are not normally in contact, Nef may modulate the activation status of the cell. It is also possible that the reported Nef functions in interference of cell signaling may be secondary to other Nef functions such as class I MHC downregulation [46].

3.4. Conservation of the multiple Nef regions responsible for downregulation of CD4 and class I MHC

Although initial reports have attributed the D/EX-

Table 1
Clinical course of hemophiliac patients studied and HIV-1 virus isolates

Date of virus Isolation	CDC classification	CD4 count (per μl)	CD4/CD8 ratio	Anti-HIV treatment	Designation of *nef* clones
Patient A:					
1989.1	2	305	0.28	none	A-I-1 to A-I-13
1989.5	2	230	0.22	none	A-II-1 to A-II-7
1990.8	2	116	0.17	none	A-III-1 to A-III-10
1994.8	4	5	N/A	AZT (92.8 to 94.2) ddC (94.2 to 94.7) AZT (94.7 to 94.8)	A-IV-1 to A-IV-7
Patient B:					
1990.7	2	585	0.80	none	B-I-1 to B-I-8
1991.2	2	660	0.68	none	B-II-1 to B-II-10
1994.9	4	60	N/A	AZT (94.4 to 94.9)	B-III-1 to B-III-9

Clone designation is given on the right, indicating the patient, the time point of virus isolation and the clone number. N/A: not available.

$XXXLL_{165}$ region responsible for CD4 downregulation [20], it was not conserved in our study. However, recent studies have identified more restricted amino acid residues, located separately in multiple locations in Nef protein, are responsible for CD4 downregulation. For example, an NMR study by Grzesiek, et al., [10] implicated that the residues WLE_{59}, GGL_{97}, R_{106} and L_{110} of Nef were involved in binding to CD4, which was recently confirmed by site-directed mutagenesis [21]. In addition, Piguet et al. [22] has recently identified EE_{155} and LL_{165} to be responsible for the Nef-mediated CD4 downregulation through its interaction with the β-subunit of COPI coatmer and adapter protein complex of clathrin-coated pits, respectively. We found that most of these sequences were well conserved in both patients.

Similarly, the Nef regions implicated in downregulation of class I MHC were also conserved. Greenberg et al [46] have reported that the N-terminal acidic region and the $(PXXP)_3$ motifs were involved in downregulation of class I MHC. Similarly, the N-terminal acidic region (at positions 62–65), also implicated in class I MHC downregulation, was conserved for its acidic nature since the observed substitutions were among acidic amino acid residues.

3.5. Concluding remarks

We have initiated this study to verify the previous functional studies of Nef using in vitro systems reported by others, since some functions of Nef delineated in vitro may not be relevant for in vivo pathogenicity. We have analyzed the *nef* gene sequences originated only from replication competent HIV-1 isolates in order to avoid sequence information derived from defective HIV-1 clones that are no longer responsible for pathogenesis. Our findings of the conservation of certain functional regions in Nef suggested that Nef is involved in AIDS pathogenesis through downregulation of CD4 and class I MHC and possibly through interference of T-cell activation signaling.

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References

[1] Cullen, B.R. (1998) Cell 93, 685–692.
[2] Kestler, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D. and Desrosiers, R.C. (1991) Cell 65, 651–662.
[3] Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C., Lawson, V.A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J.S., Cunningham, A., Dwyer, D., Dowton, D. and Mills, J. (1995) Science 270, 988–991.
[4] Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L. and Desrosiers, R.C. (1995) N. Engl. J. Med. 332, 228–232.
[5] Mariani, R., Kirchhoff, F., Greenough, T.C., Sullivan, J.L., Desrosiers, R.C. and Skowronski, J. (1996) J. Virol. 70, 7752–7764.
[6] Saksela, K., Cheng, G. and Baltimore, D. (1995) EMBO J. 14, 484–491.
[7] Lee, C.H., Leung, B., Lemmon, M.A., Zheng, J., Cowburn, D., Kuriyan, J. and Saksela, K. (1995) EMBO J. 14, 5006–5015.
[8] Collette, Y., Dutartre, H., Benziane, A., Ramos-Morales. Benarous, R., Harris, M. and Olive, D. (1996) J. Biol. Chem. 271, 6333–6641.
[9] Nunn, M.F. and Marsh, J.W. (1996) J. Virol. 70, 6157–6161.
[10] Grzesiek, S., Stahl, S.J., Wingfield, P.T. and Bax, A. (1996) Biochemistry 35, 10256–10261.
[11] Fackler, O.T., Luo, W., Geyer, M., Alberts, A.S. and Peterlin, B.M. (1999) Mol. Cell 3, 723–739.
[12] Guy, B., Riviere, Y., Dott, K., Regnault, A. and Kieny, M.P. (1990) Virology 176, 413–425.
[13] Mariani, R. and Skowronski, J. (1993) Proc. Natl. Acad. Sci. USA 90, 5549–5553.
[14] Baur, A.S., Sawai, E.T., Dazin, P., Fantl, W.J., Cheng-Mayer, C. and Peterlin, B.M. (1994) Immunity 1, 373–384.
[15] Goldsmith, M.A., Warmerdam, M.T., Atchison, R.E., Miller, M.D. and Greene, W.C. (1995) J. Virol. 69, 4112–4121.
[16] Spina, C.A., Kwoh, T.J., Chowers, M.Y., Guatelli, J.C. and Richman, D.D. (1994) J. Exp. Med. 179, 115–123.
[17] Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E. and Trono, D. (1994) Cell 76, 853–864.
[18] Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F. and Heard, J.M. (1996) Nature Med. 2, 338–342.
[19] Lu, X., Yu, H., Liu, S.H., Brodsky, F.M. and Peterlin, B.M. (1998) Immunity 8, 647–656.
[20] Craig, H.M., Pandori, M.W. and Guatelli, J.C. (1998) Proc. Natl. Acad. Sci. USA 95, 11229–11234.
[21] Mangasarian, A., Piguet, V., Wang, J.K., Chen, Y.L. and Trono, D. (1999) J. Virol. 73, 1964–1973.
[22] Piguet, V., Gu, F., Foti, M., Demaurex, N., Grueberg, J., Carpentier, J.L. and Trono, D. (1999) Cell 97, 63–73.
[23] Yamada, O., Matsumoto, T., Sasaoka, R. and Kurimura, T. (1990) AIDS 4, 35–40.
[24] Yamada, O., Kurimura, T., Wasi, C., Thongcharoen, P., Tansuphaswadikul, S. and Hoontongkam, C. (1992) Asian Med. J. 35, 225–232.
[25] Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stempien, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A., Dina, D. and Luciw, P.A. (1985) Science 227, 484–492.
[26] Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985) Cell 40, 9–17.
[27] Ina, Y. (1994) Comp. Appl. Biosci. 10, 11–12.
[28] Gojobori, T., Ishii, K. and Nei, M. (1982) J. Mol. Evol. 18, 414–423.
[29] Nakano, T., Morozumi, H., Inuzuka, S., Nagata, M., Taguchi, Y., Mizokami, M. and Okamoto, T. (1997) AIDS Res. Hum. Retroviruses 13, 563–573.
[30] Saitou, N. and Nei, M. (1987) Mol. Biol. Evol. 4, 406–425.
[31] Felsenstein, J. (1985) Evolution 39, 783–791.
[32] Felsenstein, J. (1988) Annu. Rev. Genet. 22, 521–565.
[33] Delassus, S., Cheynier, R. and Wain-Hobson, S. (1991) J. Virol. 65, 225–231.
[34] Huang, Y., Zhang, L. and Ho, D.D. (1995) J. Virol. 69, 93–100.
[35] Plikat, U., Nieselt-Struwe, K. and Meyerhans, A. (1997) J. Virol. 71, 4233–4240.
[36] Watanabe, H., Shiratori, T., Shoji, H., Miyatake, S., Okazaki, Y., Ikuta, K., Sato, T. and Saito, T. (1997) Biochem. Biophys. Res. Commun. 238, 234–239.
[37] Holmes, E.C., Zhang, L.Q., Simmonds, P., Ludlam, C.A. and Brown, A.J. (1992) Proc. Natl. Acad. Sci. USA 89, 4835–4839.
[38] Pedroza, M.L., Chenciner, N. and Wain-Hobson, S. (1992) Virology 191, 837–845.
[39] Ratner, L., Joseph, T., Bandres, J., Ghosh, S., Vander, H.N., Templeton, A., Hahn, B., Powderly, W. and Arens, M. (1996) Virology 223, 245–250.
[40] Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B. and Wain-Hobson, S. (1989) Cell 58, 901–910.
[41] Li, Y., Kappes, J.C., Conway, J,A., Price, R.W., Shaw, G.M. and Hahn, B.H. (1991) J. Virol. 65, 3973–3985.
[42] Cao, Y., Qin, L., Zhang, L., Safrit, J. and Ho, D.D. (1995) N. Engl. J. Med. 332, 201–208.
[43] Sanchez, G., Xu, X., Chermann, J.C. and Hirsch, I. (1997) J. Virol. 71, 2233–2240.
[44] Yu, G. and Felsted, R.L. (1992) Virology 187, 46–55.
[45] Aldrovandi, G.M., Gao, L., Bristol, G. and Zack, J.A. (1998) J. Virol. 72, 7032–7039.
[46] Greenberg, M.E., Iafrate, A.J. and Skowronski, J. (1998) EMBO J. 17, 2777–2889.