Intra- and Intersubtype Alternative Pak2-Activating Structural Motifs of Human Immunodeficiency Virus Type 1 Nef

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The design of antiviral strategies against human immunodeficiency virus type 1 (HIV-1) has been largely derived from studies of subtype B viruses, although they constitute only 12% of infections worldwide. At 50% of all HIV-1 infections worldwide, subtype C viruses are the most predominant. Here, we present evidence that subtype C Nefs display functional Pak2-activating motifs that differ from those found in subtype B and E Nefs. The identification of multiple Pak2-activating structural motifs that singly affect one Nef activity revealed a functional plasticity that has implications for future drug and vaccine design aimed at HIV-1 Nef and its effects on the deregulation of the immune system.

Nef is a key pathogenic factor for both human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). This was initially recognized when the infection of rhesus macaques with SIV deleted in Nef progressed to AIDS only after reversion in vivo (21). Later, a cohort of long-term nonprogressor patients and a hemophiliac patient who received a blood transfusion from an infected individual were identified to harbor viruses with deletions within the Nef gene (11, 22). Thus, intense efforts have been placed towards the study of Nef, resulting in a wealth of information regarding its function (3, 17, 46).

There are nine subtypes of HIV-1 and 15 circulating recombinant forms, with Nefs varying considerably from subtype to subtype (19, 37). In fact, Nef is one of the most variable proteins of HIV, being second only to envelope. Furthermore, SIV Nefs, which share virtually all in vitro activities of HIV Nefs, are even more divergent (5, 9, 13, 39, 41, 42). Nef demonstrates several in vitro activities believed to be central to its pathogenic role in infection. They include enhancement of virus particle infectivity, modulation of cell surface molecules (e.g., CD4, major histocompatibility class I [MHC-I], and CD74), and the activation of the cellular p21-activated protein kinase 2 (Pak2) (3, 46). It has been postulated that the complex biology of Nef is regulated through conformational changes induced by cellular location and by specific interactions with other cellular proteins involved in intracellular trafficking (2). How this mechanism of regulation is maintained in an ever-changing protein of only 27 kDa is an important biological question that remains unresolved.

Functional exchange of alternative PASM motifs between Nefs from different subtypes. We recently identified a surface on HIV-1 Nef that is specific for one of the multiple in vitro activities of Nef, the activation of Pak2 (1, 33). This surface is composed of at least four amino acids exhibiting a tendency to change in conjunction with the ability of Nef to activate Pak2.

These covariant amino acids (85, 89, 188, and 191, numbered according to reference 33) are within 6 to 10 Å of each other and form what could be a binding pocket that is critical for the stability and perhaps even for binding to a member of the Nef-Pak2 complex (1, 33). Nef consensus sequences from HIV-1 subtypes B and E encode V₈₅H₈₉R₁₈₈F₁₉₁ and F₈₅F₈₉A₁₈₈R₁₉₁, respectively, at those positions. Differences in amino acid residues surrounding this pocket could be tolerated by the properties of the residues introduced (hydrophobicity, charge, etc.) and the covariance of nearby residues. Even though certain substitutions, such as those present in the prototypic subtype B Nef encoded by HIV-1_{SF2} (L₈₅H₈₉K₁₈₈F₁₉₁), are functional, in general, subtype B or E Nef mutants with

FIG. 1. Residues A188 and H188 are components of the F₈₅F₈₉A₁₈₈R₁₉₁ and F₈₅F₈₉H₁₈₈H₁₉₁ alternative Pak2-activating structural motifs. The indicated amino acid substitutions (top) were introduced into SF2 Nef by PCR-based mutagenesis and cloned into pCDNA3.1 and the resultant plasmids transfected into 293T cells. Lysates were immunoprecipitated with anti-Nef antiserum. (Middle) Immunocomplexes were subjected to an in vitro kinase assay as previously described (4, 14, 33, 47). (Bottom) Western blot analysis of Nef proteins present in cell lysates. Activation (*n*-fold) over that of SF2 Nef is indicated for one representative experiment out of three. Ctrl, control (empty-vector-transfected cells).

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FIG. 2. Subtype B Nef isolates harboring alternative PASMs activate Pak2. (A) The amino acid compositions of chemically synthesized primary subtype B Nef isolates (1R3 and L588) or SF2 Nef-derived mutants derived by PCR-based mutagenesis are indicated (top). Nef genes were cloned and transfected as described for Fig. 1. Lysates were immunoprecipitated with anti-Nef antiserum. (Middle) Immunocomplexes were subjected to an in vitro kinase assay as previously described (4, 14, 33, 47). (Bottom) Western blot analysis of Nef proteins present in cell lysates. Asterisks denote synthetic Nef isolates 1R3 (AF120749) and L588 (AF082375). Ctrl, control (empty-vector-transfected cells). Activation (*n*-fold) over that of SF2 Nef is indicated for one representative experiment out of two. (B) HIV-1 subtype B Nef isolates or SF2 Nef mutants were cloned into pLXSN before transduction of CEM cells and staining for analysis of cell surface CD4 and MHC class I levels as previously described (33). The percentage of total cells for each quadrant is shown in each lower-right corner. Results for one representative experiment out of two are shown. (C) Patient isolate (1R3 and L588) Nef amino acid sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw>) and compared to the SF2 sequence and a subtype B consensus sequence (33). Only the residues differing from those in the consensus sequence are shown. Gaps or deletions are represented by dashes. Note the four-amino-acid duplication in SF2 Nef (R₂₂AEP₂₅) and the five-amino-acid deletion in isolate 1R3 (K₁₅₂VEEA₁₅₆), which explain their different electrophoretic mobilities.

corresponding single-amino-acid substitutions at these sites fail to activate Pak2 (33). For example, the product of the single subtype B to subtype E F191R mutation in SF2 Nef was completely devoid of the ability to activate Pak2 (33). However, when the remaining three amino acids were exchanged for those present in subtype E Nef, the resulting Pak2-activating structural motif (PASM) was found to be fully functional (33). Thus, introduction of an F₈₅F₈₉A₁₈₈R₁₉₁ subtype E PASM into subtype B SF2 Nef was fully functional (33).

Subtypes E and C are highly prevalent viruses in southeast Asia and Africa (45). Their Nefs contain different PASMs (F₈₅F₈₉A₁₈₈R₁₉₁ and F₈₅F₈₉H₁₈₈H₁₉₁) (31, 33) that when substituted into the prototypic subtype B SF2 Nef result in func-

tional proteins (33). To fully define the identities of these two PASMs as distinct motifs, we evaluated the specific contribution of the amino acid present at position 188. By exchanging the alanine and histidine at this position, we evaluated the F₈₅F₈₉H₁₈₈R₁₉₁ and F₈₅F₈₉A₁₈₈H₁₉₁ PASMs in the context of HIV-1_{SF2} Nef. As can be seen in Fig. 1, SF2-derived Nefs harboring the new motifs had reduced abilities to activate Pak2 compared to SF2-derived Nefs harboring F₈₅F₈₉A₁₈₈R₁₉₁ and F₈₅F₈₉H₁₈₈H₁₉₁ (66% ± 21% and 44% ± 12% reduction, respectively [three experiments]). These results highlight the contribution of the residue present at position 188 to the activation of Pak2 in the context of alternative PASMs. These data also define F₈₅F₈₉A₁₈₈R₁₉₁ (prevalent in subtype E Nefs)

and F₈₅F₈₉H₁₈₈H₁₉₁ (prevalent in subtype C Nefs) as alternative Pak2-activating structural motifs specific to these highly relevant subtypes.

Subtype B primary Nef isolates harbor alternative Pak2-activating structural motifs that are highly prevalent in subtypes C and E. We previously showed that certain subtype B Nefs have an H or a Y at position 191, albeit with relatively low frequencies (both 1.3%) (33). When we substituted H or Y in place of F191 in SF2 Nef, Pak2 activation was significantly reduced. However, Pak2 activation could be restored by also substituting F₈₅F₈₉H₁₈₈ or R₈₅F₈₉, respectively (33). To investigate the relevance of these results, we searched an extensive database of subtype B Nef sequences (33) to identify primary Nef isolates that contained F₈₅F₈₉H₁₈₈ or R₈₅F₈₉. Two different isolates from long-term nonprogressor patients that contain these PASM were found, L588 and 1R3 (GenBank accession numbers AF082375 and AF120749, respectively) (7, 44). Since no molecular clones containing these genes were available, they were chemically synthesized (Blue Heron, Bothell, WA) and analyzed for the ability to activate Pak2. These two Nefs activated Pak2. The levels of Pak2 activation were higher for 1R3 Nef and lower for L588 Nef than those of SF2 laboratory isolate Nef (Fig. 2A, compare lanes 5 and 8 to lane 2). These results confirm that the naturally occurring PASM F₈₅F₈₉H₁₈₈H₁₉₁ and R₈₅F₈₉R₁₈₈Y₁₉₁ are functional alternative motifs. Furthermore, these results suggest that the amino acid changes present in laboratory Nef isolates are not the result of in vitro culture but rather a property conserved in primary patient isolates.

Our original observations indicated that amino acid substitutions at positions 85, 89, 188, and 191 were highly specific for Pak2 activation. Therefore, we set out to determine whether the introduction of either a single substitution (F191H or F191Y) or triple or quadruple substitutions (L85F/H89F/K188H/F191H or L85R/H89F/F191Y) would affect other Nef in vitro activities within the context of SF2 Nef. For this purpose, we subcloned these Nefs into the retrovirus vector pLXSN, which encodes a neomycin phosphotransferase gene expression cassette, and used it to transduce human CEM T cells as previously described (14, 33). To complement this analysis, we also evaluated the same activities for the L588 and 1R3 Nef isolates. The fluorescence-activated cell sorting analysis was done after G418 selection, at which point we verified relative Nef expression levels by Western blotting as previously described (14, 33). As can be seen in Fig. 2B, neither the single nor multiple substitutions into SF2 Nef affected its ability to downregulate CD4 and MHC-I. Interestingly, the primary iso-

FIG. 3. Analysis of subtype C Nef function. (A) HIV-1 subtype C Nef isolates from Botswana were subcloned into pcDNA3.1 as previously described (33). A plasmid expressing HA-tagged Pak2 was co-transfected into 293T cells with each of the Nef-expressing plasmids, the lysates were immunoprecipitated with anti-HA antiserum, and the immunocomplexes were subjected to an in vitro kinase assay (top) as previously described (4, 33). Western blot analysis for Nef and HA-tagged Pak2 expression is shown in the middle and lower panels, respectively. Activation (*n*-fold) over that of SF2 Nef is indicated. Results for one representative experiment out of three are shown. (B) HIV-1 subtype C Nef isolates were analyzed for cell surface CD4 and MHC class I levels as described for Fig. 2 (33). The percentage of total cells for each quadrant is shown in each lower-right corner. Ctrl, control (empty-vector-transduced cells); 74, AF443074; 93, AF443093; 97, AF443097; 98, AF443098; 110, AF443110. Results for one representative experiment out of two are shown. (C) HIV-1 subtype C Nef isolates were analyzed for the ability to upregulate CD74 expression essentially as described previously (40, 43). Dark solid line, cells transfected with Nef expression plasmids; dashed line, cells transfected with the empty vector pCGCG. The NA7 primary Nef isolate described in reference 23 was used as a control. Results for one representative experiment out of two are shown. (D) Subtype C Nef amino acid sequences from patient isolates were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw>) and compared against a subtype C consensus sequence (31). Note the five-amino-acid insertion in AF443074 Nef (R₂₆KTEP₃₀) and the E66 deletion in AF443093 Nef, which explain their different electrophoretic mobilities.

late 1R3 was defective for CD4 downregulation (Fig. 2B), which correlated with a deletion within amino acids 152 to 156 (KVEEA) (Fig. 2C) that included a diacidic motif previously linked (although controversially) to this function (18, 34). A similar five-amino-acid deletion in a defective primary isolate, when restored by Na et al. (24), also restored CD4 downregulation activity. However, we should note that Janvier et al. reported data that do not support this linkage (18). A mild defect exhibited by isolate L588 in the ability to downregulate MHC-I (11% compared to 17% in SF2 Nef) could not be attributed to any Nef region known to be involved in this activity (Fig. 2C). These results confirm that the Pak2-activating structural motif is genetically separable from other Nef functions (33) and extend the observation to subtype B primary isolates harboring functional alternative motifs that are more prevalent in other subtypes.

Subtype C Nefs display structural plasticity within their Pak2-activating structural motifs. Subtype C viruses account for 50% of all HIV-1 infections worldwide and are the pre-

TABLE 1. HIV subtype C-infected patient data^a

GenBank accession no.	Gender	Age	Clinical status	No. of cells/ μl blood		Plasma viral load
				CD4	CD8	
AF443074	M	23	TB	NA	NA	>750,000
AF443093	M	37	Asymptomatic	202	923	24,156
AF443097	M	45	Asymptomatic	175	482	172,347
AF443098	M	26	Asymptomatic	484	1,138	134,320
AF443110	M	22	Asymptomatic	583	1,316	24,347

^a Plasma viral load is measured in number of RNA copies/ml blood. M, male; TB, positive for tuberculosis; NA, data not available.

FIG. 3—Continued.

dominant species in southern and eastern Africa and parts of Asia (12, 27, 45). The extended HIV-1 subtype C consensus sequence in the Pak2-activating motif illustrates the diverse nature of amino acids at positions 85 (F, 73%; V, 20%; L, 4%; I, 3%), 89 (F, 95%; W, 4%; L, 1%), 188 (S, 39%; H, 22%; Q, 20%; L, 11%; M, 3%; R, 1%; N, 1%; E, 1%; A, 1%), and 191 (R, 73%; H, 22%; Y, 5%) (31). As shown in Table 2, the analyzed Nefs have the most common amino acids at positions 85 and 89; amino acid S, H, or M at the highly diverse position 188; and all possible amino acids at position 191. The analyzed Nef clones correspond to the near-full-length genomes obtained as dominant viral variants during short-term coculture with donors' peripheral blood mononuclear cells (31). Our previous studies on viral molecular characteristics (26, 28, 31, 32) and frequencies of the MHC class I and II HLA alleles (25, 29) showed that the analyzed Nef isolates represent the overall high viral diversity in the HIV-1 subtype C epidemic. We analyzed the Nefs from five subtype C HIV isolates obtained from asymptomatic blood donors in Botswana (Table 1) for the

ability to activate Pak2. Four of the five subtype C Nefs analyzed showed levels of Pak2 activation similar to those obtained with the prototypical subtype B SF2 Nef (Fig. 3A). Isolate AF443074 was the only Nef defective for Pak2 activation. This observation was unexpected since AF443074 Nef harbors the most prevalent PASM in subtype C Nefs, F₈₅F₈₉S₁₈₈R₁₉₁ (Table 2, consensus sequence). F₈₅F₈₉S₁₈₈R₁₉₁ is also the second-most-prevalent PASM in subtype E Nefs (33). However, AF443074 Nef is the most divergent from a consensus subtype C Nef sequence derived from a published database (http://www.aids.harvard.edu/lab_research/consensus_sequence/Nef.pdf) (Fig. 3D) (31). Analysis of the AF443074 Nef amino acid sequence revealed multiple amino acid substitutions that could account for its multiply defective phenotype (Fig. 3A to D). For example, M75, K78, and V80 within the SH3-binding domain of Nef could be disruptive since this domain has been shown to be critical for the downregulation of MHC-I, the enhancement of infectivity, and the activation of Pak2 (10, 16, 48). Isolate AF443093 showed the highest (four-

TABLE 2. Amino acid residues of alternative subtype C Nef Pak2-activating structural motifs^a

Virus sequence	Amino acid at indicated position			
	85	89	188	191
Consensus	F	F	S	R
AF443074	F	F	S	R
AF443093	F	F	H	Y
AF443097	F	F	H	R
AF443098	F	F	M	R
AF443110	F	F	H	H

^a Amino acid positions are numbered according to O'Neill et al. (33). The consensus sequence was derived from Novitsky et al. (31).

fold over that of SF2 Nef) Pak2 activity of the five subtype C Nefs studied (Fig. 3A). AF443093 contained a Y at position 191 (Table 2). This result suggests that a tyrosine at this position in the context of an F₈₅F₈₉H₁₈₈Y₁₉₁ motif could result in a subtype C Nef hyperactive for Pak2 activation.

We also characterized the abilities of subtype C Nefs to modulate cell surface CD4 and MHC-I and evaluated their abilities to upregulate cell surface CD74 (MHC-II-associated invariant chain) expression as previously described (14, 33, 40, 43). Compared to the prototype HIV-1 subtype B SF2 Nef (34% CD4 and 25% MHC-I downregulation), AF443074 Nef was found to be defective for the downmodulation of both CD4 (8%) and MHC-I (1%) (Fig. 3B). AF443097 Nef had a reduced ability to downregulate MHC-I (7%), and AF443098 (11%) was impaired for CD4 downregulation (Fig. 3B). Analysis of the amino acid sequences of these two isolates revealed that isolate AF443097 had a polyacidic motif, E₆₂EADD₆₆, with several differences from the subtype C consensus sequence motif E₆₂EADD₆₆ (Fig. 3D). This motif has previously been linked to MHC-I downregulation (6, 35). On the other hand, isolate AF443098 had an A27V substitution that we have previously shown to result in a defect in CD4 downregulation (14). All five subtype C Nef isolates induced CD74 cell surface expression (Fig. 3C). Consistent with these results, the multiply defective isolate AF443074 retained the dileucine motif L₁₆₄L₁₆₅ and the E₁₇₅D₁₇₆ residues, previously shown to be critical for the upregulation of CD74 (40, 43). Together, these results show that subtype C Nefs, despite their sequence diversity, harbor alternative functional PASM, providing yet another example of the remarkable plasticity of Nef.

While we cannot completely rule out a role in maintaining structural integrity, evidence seems to indicate that the Pak2-activating structural motif has a more active role. The number of hydrophobic residues present on a solvent exposed surface, the covariance of specific sets of residues, and the specific abrogation of Pak2 activation by a minimally disruptive F-to-Y substitution argue in favor of a binding site. Here, we demonstrate that the plasticity exhibited by the Nef Pak2-activating structural motif extends to HIV-1 subtype C, the most predominant HIV-1 subtype in the worldwide epidemic (12, 14, 27, 33). Blocking the positive effect of Nef on viral replication, its direct pathogenicity, or its immune evasion effects would potentially have great therapeutic synergy when combined with highly active antiretroviral therapy (38). The number of functional alternative PASM identified so far (e.g., L₈₅H₈₉K₁₈₈F₁₉₁, F₈₅F₈₉H₁₈₈H₁₉₁, and F₈₅F₈₉A₁₈₈R₁₉₁ in sub-

type B, C, and E Nefs, respectively) suggests that when attempting to target Nef, subtype-specific antiviral strategies should be considered (17, 38). This becomes particularly important when considering that viral genetic diversity, as defined by subtype designation, may give rise to differential propensities towards antiviral resistance between viruses from different geographical locations (45). In fact, subtype C HIV-1 has been shown to have a reduced threshold for the development of resistance against tenofovir in vitro (8). To that end, it should be noted that most current antiretroviral drugs were designed after subtype B isolates, which have also been used to generate most data on mechanisms of drug resistance (20). In addition, it has been shown that amino acid pairs 85/89 and 188/191 of subtype B Nef lie within immunodominant regions in HIV that are consistently a target of cytotoxic T lymphocytes (CTLs) across multiple ethnicities (15). The amino acid pair 85/89 is also within the immunodominant regions of subtype C viruses (28). Therefore, immune pressure may not only drive viral escape but also result in the modification of the amino acids that compose the Pak2-activating structural motif. The ability of Nef to mutate amino acids within immunodominant epitopes and covary distant amino acids to restore its function and positive effects on viral replication could contribute to the high viral loads observed in the face of a strong CTL response (30). The direct correlation between viral loads and host CTL responses against Nef directly contrasts with the inverse correlation displayed by the structural and more constrained (and thus less variable) p24 Gag protein (30). Our results indicate that further investigation towards the elucidation of non-subtype B HIV protein structure-function relationships to complement the current knowledge regarding subtype B HIV proteins is warranted. Our work shows that the dynamic evolution that Nef has undergone has resulted in a remarkable functional plasticity that extends across subtypes and will impact the design of future antivirals and vaccines for HIV, particularly if other HIV proteins are found to display similar mechanisms to maintain a balance between viral fitness and viral escape.

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