Published in final edited form as:

J Mol Biol. 2009 November 27; 394(2): 329–342. doi:10.1016/j.jmb.2009.09.047.

HIV-1 Nef Dimerization is required for Nef-Mediated Receptor Downregulation and Viral Replication

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Abstract

Nef, an HIV-1 accessory factor capable of interaction with a diverse array of host cell signaling molecules, is essential for high-titer HIV replication and AIDS progression. Previous biochemical and structural studies have suggested that Nef may form homodimers and higher order oligomers in HIV-infected cells, which may be required for both immune and viral receptor downregulation as well as viral replication. Using bimolecular fluorescence complementation (BiFC), we provide the first direct evidence for Nef dimers within HIV host cells and identify the structural requirements for dimerization *in vivo*. BiFC analysis shows that the multiple hydrophobic and electrostatic interactions found within the dimerization interface of the Nef X-ray crystal structure are essential for dimerization in cells. Nef dimers localized to the plasma membrane as well as the *trans*-Golgi network, two subcellular localizations essential for Nef function. Mutations in the Nef dimerization interface dramatically reduced both Nef-induced CD4 downregulation and HIV replication. Viruses expressing dimerization-defective Nef mutants were disabled to the same extent as HIV that fails to express Nef in terms of replication. These results identify the Nef dimerization region as a potential molecular target for anti-retroviral drug discovery.

Keywords

HIV Nef; bimolecular fluorescence complementation; dimerization; CD4; HIV replication

Introduction

HIV-1 Nef is a small myristoylated HIV accessory protein essential for AIDS pathogenesis. Highly conserved among primate lentiviruses, Nef is a critical determinant of both high-titer viral replication and AIDS progression¹. In a transgenic mouse model, expression of Nef alone is sufficient to induce profound immunodeficiency and CD4⁺ T-cell depletion, identifying this accessory protein as a key determinant of HIV pathogenesis^{2, 3}. Conversely, HIV strains with defective *nef* alleles have been isolated from patients with long-term, non-progressive HIV infection, further implicating Nef as a critical virulence factor for AIDS^{4, 5}.

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Despite the lack of catalytic activity, Nef influences numerous signaling pathways within the infected host cell. Nef enhances viral replication and disease progression by altering the threshold of T-cell activation^{6–8}, influencing transcriptional and cellular activation^{3, 9–11}, enhancing virion infectivity^{12–15} and allowing escape of HIV-infected cells from immune surveillance through downregulation of cell-surface MHC-I molecules^{16–19}. Perhaps the best characterized function of Nef is its ability to reduce the steady state levels of CD4 on the host cell surface^{20–23}. This rapid downregulation of CD4 by Nef prevents viral superinfection as well as sequestration of viral progeny^{24, 25}. Indeed, HIV replicates poorly in cell lines engineered to overexpress CD4 molecules that are insensitive to Nef-mediated downregulation^{26, 27}.

Multiple Nef amino acid sequence motifs have been identified that are critical for altering the cellular activation and signaling pathways described above^{28, 29}. While regions within the flexible amino-terminal arm and central loop have been well characterized^{30–33}, the biological relevance of the structured core has not been fully investigated, especially in terms of its role in homotypic Nef:Nef interactions within a biological context. X-ray crystallography strongly suggests multiple contact points between Nef monomers, including Arg105, Ile109, Leu112, Tyr115, Phe121, and Asp123 within the α B helices of the Nef core (numbering based on the crystal coordinates of Lee et al., 1996) (Figure 1). These residues comprise a hydrophobic interface (residues Ile109 through Phe121) flanked by pairs of electrostatic interactions (formed by residues Arg105 and Asp123). The possibility exists that Nef dimers are the result of crystal packing, and may not be of biological significance. However, all of the residues that contribute to the dimerization interface are highly conserved among HIV-1 Nef isolates, strongly suggesting an essential function for dimerization *in vivo*. Indeed, mutagenesis of Asp123 has been shown to affect Nef-induced kinase activation and receptor downregulation^{34, 35}, although the impact of this mutation on Nef dimerization in HIV target cells is not completely clear.

In this study, we provide direct evidence that dimerization is critical for Nef function *in vivo*. Using a unique fluorescence-based approach known as bimolecular fluorescence complementation (BiFC)³⁶, we identified the structural requirements for Nef dimerization within HIV host cells. We found that Nef dimerization *in vivo* is very sensitive to mutations targeting the dimerization interface predicted by the crystal structure, but is independent of membrane association and the highly conserved protein-protein interaction motif, PxxPxR. BiFC analysis revealed that Nef dimers localize to the plasma membrane as well as the *trans*-Golgi network, subcellular sites essential for Nef function¹⁷. Partial or complete disruption of dimerization by mutagenesis completely prevented Nef-induced CD4 downregulation in every case, suggesting that a precise conformation of the Nef dimer is required for this essential Nef function. Furthermore, disruption of Nef dimerization reduced HIV replication to levels observed with HIV that fails to express Nef. These observations provide the first evidence the dimerization is critical to Nef-dependent enhancement of HIV replication and identifies the Nef dimerization interface as a potential target for anti-retroviral drug design.

Results

Detection of Nef dimers *in vivo* using bimolecular fluorescence complementation

In previous work, we provided evidence for Nef dimerization in live cells using bimolecular fluorescence complementation (BiFC) of YFP³⁶. For these experiments, two expression vectors were created by fusing non-fluorescent N-terminal (YN_{1–154}) or C-terminal (YC_{154–238}) fragments of YFP to HIV-1 Nef. The YFP fragments were fused to the C-terminal end of Nef in order to preserve the native N-terminal myristoylation signal sequence. Homotypic Nef:Nef interaction brings the two non-fluorescent YFP fragments into close proximity, reconstituting the functional YFP structure (Figure 2A). Co-expression of the Nef-

YN and Nef-YC fragments in 293T cells results in a strong fluorescent signal as detected by confocal microscopy (Figure 2B, top right).

To confirm that the BiFC signals observed require Nef dimerization, control BiFC expression plasmids were generated using glutathione *S*-transferase (GST). GST does not interact with Nef³⁷, yet is a dimeric protein in its own right and serves as an internal positive control. GST-based BiFC expression vectors were paired with the complementary Nef-BiFC counterparts (e.g. GST-YN + Nef-YC and GST-YC + Nef-YN) and co-expressed in 293T cells. As shown in Figure 2B (top row), co-expression of the GST and Nef BiFC pairs did not produce a fluorescent BiFC signal. In contrast, co-expression of GST-YN and GST-YC produced a strong BiFC signal consistent with GST dimerization. To control for expression of the GST and Nef BiFC constructs, transfected cells were immunostained with GST and Nef antibodies. These control experiments demonstrate that despite high-level co-expression, the mere presence of the YN and YC fragments cannot drive fluorescence complementation. Taken together, these data support the conclusion that the Nef BiFC signal is strictly dependent upon Nef dimerization *in vivo*.

We next expanded our study of Nef dimerization using cell lines that support HIV replication, including the U87MG astrogloma and SupT1 T-lymphoblast cell lines. However, unlike the 293T cells previously used in the experiments described above, these cell lines did not tolerate the ~3 hour incubation at room temperature required to promote maturation of the YFP fluorophore. To overcome this problem, we used the newly described GFP variant, Venus, which yields a BiFC signal without a requirement for room temperature incubation³⁸. The Nef coding sequence was therefore fused to Venus N- and C-terminal fragments and the Nef BiFC partners were introduced into each of these cell lines using retroviral vectors. BiFC signals were readily observed in each case (Figure 3A), demonstrating that Nef dimerizes in a variety of host cell environments and supports the conclusion that dimerization is an intrinsic property of Nef.

Subcellular localization of Nef dimers

Nef is targeted to cellular membranes via N-terminal myristoylation of a conserved Met-Gly-X-X-X-Ser/Thr motif^{39, 40}, suggesting that plasma membrane localization and clustering in lipid rafts may promote dimerization. To test this idea, a point mutation (G2A) was introduced into the Nef myristoylation signal sequence to prevent myristoylation. BiFC fusion partners were then generated from the Nef-G2A mutant, expressed in 293T cells and assayed for dimerization. As shown in Figure 3B, Nef-G2A produced a strong BiFC signal, indicative of dimerization. However, the G2A mutant displayed exclusively cytoplasmic fluorescence, and lacked the membrane localization observed with wild-type Nef. These results suggest that Nef dimerization is independent of membrane localization.

Nef has been shown to bind with high affinity to the SH3 domains of Src-family kinases and other proteins via its conserved PxxPxR motif^{33, 41, 42}. The Nef mutant 2PA, in which the key proline residues within this motif are replaced with alanines, was used to examine the requirement for SH3 partner proteins in Nef dimerization. As shown in Figure 3B, disruption of the Nef SH3-binding motif had no effect on the BiFC signal or its subcellular localization as compared to wild-type Nef. This finding suggests that Nef dimerization does not require interactions with other proteins through the PxxPxR motif, despite the presence of SH3 domains in the dimeric Nef X-ray crystal structures^{31, 33}.

Other studies have shown that Nef also localizes to the *trans*-Golgi network (TGN) where it interacts with multiple trafficking and signaling proteins^{43, 44}. We observed a consistent subpopulation of Nef BiFC-positive cells with peri-nuclear fluorescence, suggesting that Nef dimers localize to the TGN. To determine if this was the case, cells were counterstained with

antibodies to TGN-46, a marker for this subcellular compartment. As shown in Figure 3C, co-localization of the Nef BiFC signal and TGN-46 immunostaining was observed, indicating that Nef dimers localize to the TGN in 293T cells. A similar peri-nuclear Nef-BiFC signal was also observed in U87MG and SupT1 cells, suggesting that localization to the TGN is a general characteristic of Nef dimers and not specific to a particular cell type (Figure 3A).

Efficiency of Nef dimerization

As a prelude to mutagenesis of the Nef dimerization interface, we next examined the efficiency with which Nef dimerization can be detected using the BiFC approach. Relative Nef expression levels were detected in 293T cells expressing Nef-YN and Nef-YC by indirect immunofluorescence (IF). This approach permitted a direct correlation between the number of cells expressing Nef and the number of cells in which Nef dimerization had occurred. As shown in Figure 4A, detection of Nef dimerization using BiFC is highly efficient, with a positive BiFC signal present in virtually every cell positive for Nef expression by IF. We then determined the pixel intensities of the Nef immunofluorescence and BiFC signals for 100 individual cells using ImageJ. This analysis revealed that a positive linear correlation exists between BiFC signal intensity and the level of Nef expression (Figure 4B). These data demonstrate that BiFC provides a highly efficient method to detect Nef dimerization in cells, and therefore provides a useful model system to probe the dimer interface by site-directed mutagenesis as described in the next section.

Probing the Nef dimerization interface by BiFC

Previous structural studies indicate that multiple contact points of highly conserved amino acids define the Nef dimerization interface^{31, 33}. These contacts are principally established between the α B helices, which form a hydrophobic core consisting of conserved residues Ile190, Leu112, Tyr115, and Phe121 flanked on either end by ion pairs formed between residues Arg105 and Asp123 (Figure 1). To test the contributions of these hydrophobic and ionic interactions to Nef dimerization in cells, these residues were systematically disrupted by site-directed mutagenesis and their effects on dimerization were assayed via BiFC. In order to scan the hydrophobic interface (Figure 1B), changes were introduced with single (L112A, F121A, L112D, Y115D), double (L112D/Y115D) or quadruple (I109D/L112D/Y115D/F121D) mutations. Single and double mutations were also created in the ion pairs flanking the hydrophobic core (D123A, D123N, D123V, R105E, and R105E/R106E; Figure 1C). Secondary structure analysis revealed that these mutations should not disrupt the helical nature of this region of the Nef core (Supplemental Figure S1). These Nef mutants were expressed as pair-wise BiFC constructs in 293T cells and counterstained with an anti-Nef antibody to verify mutant protein expression. The transfected cell populations were then imaged for dimerization (BiFC) and Nef expression (IF) and the BiFC:IF ratio was calculated using the Metamorph software package. As shown in Figure 5, Table 1, and Supplementary Figure S2, single and double amino acid changes designed to disrupt the hydrophobic interface significantly reduced but did not abolish the Nef BiFC signal. However, substitution of all four hydrophobic interface residues with aspartate (Nef-4D mutant) completely abolished BiFC, demonstrating that an intact hydrophobic interface is essential for dimerization *in vivo*. Similarly, disruption of the intermolecular ion pair between Asp-123 and Arg-105 by substitution of Asp-123 with asparagine completely abolished BiFC, identifying this residue as a key modulator of dimerization. However, substitution of Asp-123 with alanine or valine as well as substitution of Arg-105 with aspartate reduced but did not abolish Nef BiFC, suggesting that these mutations destabilize but do not completely disrupt the Nef dimer.

An intact dimerization interface is required for Nef-induced CD4 downregulation

Downregulation of cell surface CD4, the primary receptor for HIV, is one of the best-known functions of Nef (see Introduction). Based on previous observations that some Nef residues critical for CD4 downregulation are located within the dimerization interface as defined by the crystal structure³⁴, we explored the requirement for Nef dimerization in CD4 downmodulation using our panel of Nef mutants identified by BiFC. SupT1 cells were infected with recombinant retroviruses carrying wild-type or mutant forms of Nef, and cell-surface CD4 levels were assessed via flow cytometry. As shown in Figure 6A, wild-type Nef induced marked downregulation of CD4 relative to the uninfected control cell population. In contrast, the dimerization-defective Nef mutants 4D and D123N completely failed to downregulate CD4 from the cell surface. In fact, all mutations in the dimerization interface compromised Nef-induced CD4 downregulation, despite partial dimerization of these Nef mutants as judged by BiFC (compare Figure 6B and Table 1). These data provide the first direct correlation between Nef dimerization and CD4 downregulation in the same HIV-1 host cell population, and demonstrate that Nef must adopt the wild-type, dimeric state to induce CD4 downregulation. Expression of wild-type and mutant Nef protein expression in the transduced SupT1 population was verified via immunoblotting (Figure 6C). Equivalent levels of Nef immunostaining were observed, with the exception of the Nef-4D mutant which was somewhat reduced. This difference is most likely due to disruption of the epitope for antibody recognition, which partially overlaps with the site of these mutations.

The myristoylation-defective Nef-G2A mutant served as a control for the extent of Nef-induced CD4 downregulation, as previous studies have established that membrane targeting is essential for this Nef function⁴⁵. As shown in Figure 6B, the extent of CD4 downregulation by the dimerization-defective Nef mutants was comparable to that observed with Nef-G2A despite membrane localization of all Nef mutants as confirmed by IF staining (Figure 5 and Supplemental Figure S1). These results demonstrate that localization of Nef to the plasma membrane alone is insufficient for CD4 downregulation, and implicate dimerization as a critical property of Nef required for the removal of CD4 from the surface of HIV-infected cells.

In order to ensure that the loss of CD4 downregulation was not due to misfolding of the Nef core as a result of the mutations, SH3 domain-binding activity was assessed for several of the dimerization-defective mutants. Engagement of Nef by the SH3 domain of the Src-family kinase Hck requires not only the Nef PxxPxR motif, but is also dependent upon the three-dimensional fold of the Nef core which creates a binding pocket for the RT loop of the SH3 domain^{37, 46}. Recombinant immobilized GST-SH3 fusion proteins were incubated with purified recombinant wild-type and mutant Nef proteins. As shown in Figure 7, all four of dimerization-defective Nef mutants tested bound to the Hck SH3 domain, indicating that mutations in the Nef dimerization interface do not influence the overall fold of the Nef core. No binding was observed with a nonfunctional SH3 domain mutant (W93A), which served as a negative control^{47, 48}.

Nef dimerization is required for HIV replication

Results presented in the previous section demonstrate that Nef dimerization is required for CD4 downregulation. Because removal of CD4 from the host cell surface is critical for optimal HIV replication and pathogenesis^{49, 50}, we next investigated the requirement for Nef dimerization in HIV replication. For these experiments, each of the Nef mutants evaluated for dimerization in the BiFC assay were introduced into the Nef coding region of the HIV_{NL4-3} provirus. Viral replication was then investigated using the astrogloma cell line U87MG, which has been modified to express CD4 and the co-receptor CXCR4^{51–53}. These cells provide a useful system for analysis of Nef-dependent HIV replication, as an HIV-1 mutant that fails to express Nef replicates poorly in this cell line (Emert-Sedak, L. and Smithgall, T.E., manuscript

submitted; Figure 8 and Supplemental Figure S3). U87MG/CXCR4/CD4 cells were infected with equivalent titers of wild-type or mutant Nef viruses, and the extent of viral replication was monitored by p24 ELISA 4 days later. As shown in Figure 8, replication of HIV carrying the dimerization-defective Nef mutants was reduced to an equivalent extent as the virus that fails to express Nef (Δ Nef). Expression of Nef from the wild-type and mutant proviruses was confirmed by immunoblotting. These data show for the first time that Nef must exist in a wild-type, dimeric state in order to enhance HIV replication.

Discussion

In this study, we used YFP-BiFC analysis of Nef mutants to identify structural features essential for Nef dimerization in live cells. Using this approach, we show that residues that form the dimerization interface in the X-ray crystal structures of Nef are essential for Nef dimerization and function in cell-based assays. Most unexpected were the profound effects of Nef dimerization interface mutations on HIV replication, identifying this region of Nef as an unexplored drug discovery target.

To confirm that fluorescence complementation was dependent upon Nef:Nef interaction, we created control BiFC constructs using GST. Importantly, BiFC was not observed when the control GST BiFC fusion proteins were co-expressed with the complementary Nef BiFC proteins. The GST BiFC fusion proteins also served as an internal control for the assay, as co-expression of GST-YN with GST-YC resulted in a strong BiFC signal. The subcellular localization of the GST and Nef dimers, as reported by BiFC, were also quite distinct, with GST dimers localizing to the cytoplasm and Nef dimers localizing to the plasma membrane and TGN. These findings demonstrate that BiFC accurately reflects the subcellular localization of Nef dimers within the cellular environment, consistent with recent reports that applied this technique to study of other dimeric proteins^{54–57}.

Previous studies have shown the significance of membrane localization for Nef function^{32, 45, 58}. These findings led us to examine if membrane localization was required for dimerization, possibly due to Nef clustering in lipid rafts⁵⁹. However, we observed that a myristoylation-defective mutant of Nef (G2A) produced a diffuse cytoplasmic BiFC signal, indicating that membrane attachment is not required for the formation of Nef dimers. These findings are consistent with previous studies of Arold et al., who demonstrated that a purified recombinant Nef core domain formed homodimers in vitro⁶⁰.

Nef interacts with multiple binding partners in HIV-infected cells, suggesting that binding of host cell proteins may influence Nef dimerization in vivo. The highly conserved PxxPxR motif is well-known to mediate Nef interactions with partner proteins that have SH3 domains, including Src-family kinases and guanine nucleotide exchange factors, as well as other signaling proteins^{11, 32, 61–64}. However, mutation of the essential prolines in this motif did not diminish the BiFC signal nor did it influence the subcellular localization of Nef. This observation suggests that interactions with other factors, at least through the PxxPxR motif, are not essential for Nef dimerization. This finding is consistent with the observation that the Nef SH3-binding motif is spatially separated from the dimerization interface in the crystal structure.

In addition to providing information on protein-protein interaction in cells, BiFC also reports the subcellular localization of the dimers. Our experiments revealed consistent localization of Nef BiFC signals to the plasma membrane and perinuclear region in all three cell types examined (293T epithelial cells, U87MG astrogloma cells, and SupT1 T-cells). The perinuclear localization of the Nef dimers was coincident with immunofluorescent staining for the TGN marker protein, TGN-46. These observations are consistent with previous studies

showing that localization of Nef to the TGN is essential for MHC downregulation^{17, 43}, and suggest that Nef dimerization may be essential for MHC downregulation as well. Indeed, previous work by Liu et al. shows that mutation of Asp 123, shown here to completely quench the Nef-BiFC signal, interferes with Nef-induced MHC downregulation³⁴.

Previous X-ray crystallography studies have identified residues within the Nef core that define a dimerization interface^{31, 33}. Highly conserved among isolates of both HIV and SIV Nef, these residues contribute to dimerization through a combination of hydrophobic and electrostatic interactions. Using the BiFC assay and site-directed mutagenesis, we demonstrate that the dimerization interface defined by crystallography is indeed essential for Nef dimerization in cells. Both an intact hydrophobic core and the flanking ionic interactions are essential for maintaining Nef dimerization and function *in vivo*. A complete loss of the Nef-BiFC signal was observed when the four key residues that form the hydrophobic interface were replaced with charged residues (Nef-4D) or when Asp 123, which ion pairs with Arg 105, was replaced with asparagine. Interestingly, replacement of Asp 123 with the nonpolar residues valine or alanine reduced but did not abolish BiFC, suggesting that these mutants still retained the capacity to dimerize, albeit with severe functional consequences in terms of CD4 downregulation and HIV replication (see below). The differences observed with these Asp 123 mutants may be a function of the irreversible nature of the BiFC assay; that is, once YFP complementation has taken place, the reformed fluorophore is extremely stable and does not dissociate. The D123N mutation must raise the K_D for homotypic Nef interactions above a critical threshold required to elicit complementation⁶⁵, and therefore scores as negative in the BiFC assay. On the other hand, the D123A and D123V mutants must reduce Nef:Nef interaction affinity as complementation efficiency is significantly lower than wild-type, but not to the point to completely abrogate BiFC.

Defining regions of Nef critical for the induction of CD4 downregulation have been the subject of previous studies that involved mutagenesis of residues near or within the dimerization interface^{34, 42}. Results presented here demonstrate a direct correlation between residues critical for CD4 downregulation and those essential for dimerization *in vivo*. Without exception, BiFC analysis shows that all mutations within the dimerization interface significantly reduced or abolished dimerization in cells and also interfered with Nef-induced CD4 downregulation. Our observation that dimerization was disrupted to varying degrees in our panel of Nef mutants yet CD4 downregulation was completely lost suggests that full inhibition of Nef dimerization is not required to induce a loss of this Nef function.

In a final series of experiments, we demonstrate for the first time that dimerization is essential for Nef to support HIV replication in cell culture. None of the dimerization-defective Nef mutants identified in the BiFC screen supported HIV replication in U87MG cells. Comparable low levels of replication were observed between virus carrying the dimerization-defective Nef mutants and a control virus that fails to express Nef altogether. Interestingly, a functional link between Nef-induced CD4 downregulation and HIV replication has been reported^{50, 66, 67}, suggesting that the observed effects of dimerization interface mutations on CD4 downregulation and HIV replication may be related. As observed for CD4 downregulation, Nef mutants displaying either partial or complete loss of dimerization by BiFC analysis all lost their capacity to enhance HIV replication. These results indicate that Nef functions related to HIV replication are highly sensitive to structural perturbations in the dimerization interface.

Materials and Methods

Cell Culture and Reagents

The human cell lines 293T, U87MG, and SupT1 were obtained from the ATCC, and maintained at 37 °C in a humidified incubator with a 5% CO₂ atmosphere. 293T cells were cultured in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Atlanta Biological) and antibiotic-antimycotic (Invitrogen). U87MG cells were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS and 50 µg/mL gentamycin (Invitrogen). U87MG/CD4/CXCR4 cells (generous gift of Dr. Toshiaki Kodama, University of Pittsburgh School of Medicine) were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS, 300 µg/mL G418 and 0.5 µg/mL puromycin. SupT1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and antibiotic-antimycotic. 293T cells were transfected using standard calcium phosphate techniques as described elsewhere⁶⁸.

Bimolecular Fluorescence Complementation (BiFC) Expression Vectors

Two Nef-BiFC expression vectors were created in which the N-terminal coding sequence of YFP (residues Val2-Ala154) were PCR-amplified from the plasmid vector pEYFP-C1 (Clontech) and ligated into the mammalian expression vector pcDNA3.1(-) (Invitrogen) containing Nef (SF2 allele) via a unique Acc III restriction site found near the Nef C-terminal coding region to create Nef-YN. A similar approach was used to fuse Nef to the C-terminal coding region for YFP (residues Ala154-Lys238) to create Nef-YC. Mutagenesis of the N-terminal myristoylation signal sequence, the conserved PxxPxR SH3-binding motif, and the Nef dimerization interface were performed using either the QuikChange II site-directed mutagenesis kit (Stratagene) or standard PCR-based techniques.

For the Venus-based BiFC expression vectors, the coding sequences for the Venus N-terminal residues Val2-Asp173 and C-terminal residues Ala154-Lys238 were amplified by PCR and fused to Nef in pcDNA3.1(-) as described above for the Nef-YFP BiFC constructs. The Venus template was a kind gift of Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan.

To create the control BiFC-GST fusion constructs, the identical N- and C-terminal coding sequences of YFP were PCR-amplified as described above and subcloned into pcDNA3.1(+) (Invitrogen) via unique NheI/HindIII restriction sites. The coding sequence of GST was then PCR-amplified from pGEX-2T (GE Life Sciences) and ligated in-frame with YN and YC coding regions to generate pcDNA3.1(+)YN-GST and pcDNA3.1(+)YC-GST fusion plasmids. Protein expression was verified via monoclonal antibodies raised against either Nef or GST as described below.

Immunofluorescence Microscopy and Fluorescence Imaging

The antibodies used for the immunofluorescence experiments include anti-Nef (NIH AIDS Research & Reference Reagent Program; mAb Hyb 6.2; 1:500), anti-GST (Santa Cruz Biotechnology; sc-459; 1:1000), and an antibody against a *trans*-Golgi network marker (Serotec; TGN46; 1:500). Transfected 293T cells were grown on coverslips and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were washed with PBS, treated with 0.2% Triton X-100 in PBS for 15 min, and washed again with PBS. Cells were then blocked with PBS containing 2% BSA for 30 min and incubated for 45 min at room temperature with the primary antibodies diluted as described above, followed by additional washing with PBS.

Immunostained cells were then visualized with secondary antibodies conjugated to Cy3/Cy5 (Chemicon; 1:1000) or Texas Red (Southern Biotech; 1:750). BiFC and immunofluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT cooled CCD high-resolution digital camera and software (Diagnostic Instruments). Confocal multi-color images (YFP, Cy3, Cy5) were obtained using a Zeiss Meta 510 confocal microscope at the Center for Biological Imaging (CBI) at the University of Pittsburgh School of Medicine.

To quantify the effects of the mutations on BiFC, immunofluorescent and BiFC images from the same culture were quantitatively analyzed using the Metamorph software suite (Molecular Devices). The lower threshold fluorescence intensities for all BiFC images were standardized using BiFC control plasmids, as per published protocols⁶⁹. Upper threshold fluorescence intensities were then standardized to wild type Nef-BiFC and the corresponding immunofluorescent image such that the final output was based on total threshold area and staining intensity at the individual pixel level. Mutant Nef-BiFC levels were then measured relative to the standards established above.

Recombinant Retroviruses for BiFC Assays

Retroviral expression vectors were used to generate high-titer retroviral stocks in 293T cells by cotransfection with an amphotropic packaging vector as described elsewhere³⁶. Nef-Venus fusion constructs were subcloned into the retroviral expression vector pSR α MSVtkneo70. Retroviral stocks were supplemented with Polybrene (Sigma) to 4 μ g/mL and added to U87MG and SupT1 cells in 6-well plates (2.5×10^5 cells/well). The plates were centrifuged at 1000g for 4 h at 18 °C to enhance infection efficiency. Because the BiFC system requires the co-expression of two Nef fusion proteins (Nef-VN and Nef-VC), cells were super-infected with the second retrovirus 24 h later. Cultures were screened for BiFC 72 h later and images recorded using a Nikon TE300 inverted microscope as described above.

Retroviral Transduction of Nef Mutants and Flow Cytometric Analysis of CD4

Retroviral plasmids (pSR α MSVtkneo) containing unfused, wild-type and mutant forms of Nef were used to generate amphotropic retroviruses by co-transfection of 293T cells as described above. SupT1 cells were infected with the recombinant retroviruses, selected with G418 (800 μ g/mL) for two weeks, and Nef protein expression was confirmed by immunoblotting. Cells were stained for cell-surface CD4 in FACS medium (2% fetal bovine serum in PBS) containing an allophycocyanin (APC)-conjugated anti-human CD4 monoclonal antibody (clone RPA-T4; BD Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Pharmingen).

Expression of GST-SH3 Fusion Proteins and Nef-SH3 Binding Assay

For these experiments, coding sequences of wild-type and mutant (W93A) forms of the Hck SH3 domain were subcloned into the bacterial expression vector pGEX-2T (GE Biosciences). GST-SH3 fusion proteins were expressed in *E. coli* strain DH5 α and immobilized on glutathione-agarose beads. HIV-1 Nef-SF2 coding sequences (wild-type as well as the dimerization interface mutants shown) were subcloned into the bacterial expression vector pET14b. Recombinant Nef proteins were expressed in *E. coli* strain BL21(DE3)pLysS and purified via N-terminal His-tags^{37, 71}. Equimolar amounts of Nef (2 μ g) and immobilized GST-SH3 proteins were incubated in 500 μ L incubation buffer [50 mM Tris-HCl (pH 7.4), 2% BSA, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1% (v/v) Triton X-100] and rotated at 4°C for 2 h. Nef-SH3 complexes were pelleted, washed extensively in RIPA buffer, resolved by 12% SDS-PAGE and associated Nef was detected by immunoblotting.

HIV Replication Assays

The coding sequence for Nef was excised from the NL4-3 provirus and subcloned into pGL3-Basic (Promega) via the unique restriction sites BamHI/NcoI. A 188 bp Bsu36I/EcoRV fragment was cut from the coding region of wild-type and mutant forms of Nef used in the BiFC experiments (SF2 strain) and subcloned into the pGL3-Basic/Nef vector to generate hybrid SF2-NL4-3 Nef alleles. The coding regions of these Nef hybrids were then subcloned cloned back into the NL4-3 provirus via the same BamHI/NcoI restriction sites. The wild-type HIV-1_{NL4-3} and HIV-1_{Nef-NL4-3/SF2} proviral constructs were then transfected into 293T cells

using FuGENE6 (Roche) using the manufacturer's protocol and viral supernatants collected 48 h post-transfection. SupT1 cells (5×10^5) were infected with 200 μ L viral supernatant, and incubated for 2 h at 37 °C. Infected cells were washed with serum-free RPMI and centrifuged at $1500 \times g$ for 4 min at room temperature and resuspended in 6 mL of RPMI supplemented with 10% FBS. Upon syncytia formation (3–5 days post-infection), amplified viral supernatants were clarified by centrifugation at $1500 \times g$ for 4 min and viral titers were determined by p24 ELISA using the manufacturer's protocol (SAIC-Frederick, Inc). ELISA plates were read on a Multiscan MCC341 Microplate Reader (Finstruments) and analyzed using Spectrosoft software. Viral replication was monitored in U87MG/CD4/CXCR4 cells by infection with an equivalent initial input of 250 pg p24/mL. Control experiments show that the HIV-1_{Nef-NL4-3/SF2} hybrid virus forms syncytia and replicates in U87MG cells in the same way as wild-type HIV-1_{Nef-NL4-3} (Supplemental Figure S2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grants AI57083 and CA81398 (to T.E.S.) and by the University of Pittsburgh AIDS Research Training Grant T32 AI065380 (to J.A.P.). The authors also wish to thank Dr. Simon Watkins of the Center for Biologic Imaging, University of Pittsburgh School of Medicine, for assistance with the confocal microscopy, and the National Institutes of Health AIDS Research and Reference Reagent Program.

Abbreviations

BiFC	bimolecular fluorescence complementation
YFP	yellow fluorescent protein
TGN	<i>trans</i> -Golgi network
IF	immunofluorescence
DPI	days post infection

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Figure 1.

Crystal Structure of the Nef Dimerization Interface. (a) Two views of the X-ray crystal structure of the dimeric Nef core. The two halves of the Nef dimer are colored green and blue, respectively. The boxed region in the lower panel shows the juxtaposition of the B helices which form the dimer interface. This region is enlarged in panels (b) and (c), which show the side chains of key hydrophobic and charged residues that contribute to dimerization, respectively. These models were produced using PyMol Molecular Viewer and the X-ray crystal coordinates of Lee, et al. (**PDB: 1EFN**)³³.

Figure 2.

Nef Dimerization Drives BiFC in 293T Cells. (a) Illustration of the BiFC principle. Non-fluorescent fragments of YFP (YN and YC) are fused to interacting proteins (Nef in this case). When the Nef-YN and Nef-YC fusion proteins are co-expressed in the same cell, Nef:Nef interaction brings the YFP fragments into close proximity, resulting in functional complementation and fluorescence. (b) BiFC expression constructs were created for HIV-1 Nef (Nef-YN and Nef-YC) as well as for GST as a control (GST-YN and GST-YC) as described under Experimental Procedures. All four possible combinations of fusion proteins were co-expressed in 293T cells, and BiFC was assessed by confocal microscopy (BiFC; green; top row). Protein expression was assessed by immunostaining with anti-Nef (second row; red) and

anti-GST (blue; third row) antibodies. A merged image is shown in the bottom row. Scale bar, 25 μm .

Figure 3.

Characterization of Nef dimerization by BiFC. (a) U87MG and SupT1 cells, both of which support HIV-1 replication, were co-infected with Nef-VN and Nef-VC retroviruses and examined for Venus complementation by fluorescence microscopy. 293T cells co-transfected with the Nef-YN and Nef-YC expression constructs were included as a positive control. Scale bars 25 μ m, 50 μ m and 15 μ m, respectively. (b) Nef dimerization does not require myristoylation or interaction with SH3-binding proteins. 293T cells were co-transfected with wild-type Nef-YN and Nef-YC expression constructs (Nef-WT), with constructs bearing a Gly-2 to Ala substitution in the N-terminal myristoylation signal sequence (Nef-G2A), or Ala for Pro substitutions in the SH3-binding motif, PxxPxR (Nef-2PA). BiFC was analyzed by fluorescence microscopy 48 h later. Scale bar, 25 μ m. (c) Nef dimers localize to the *trans*-Golgi network (TGN). 293T cells co-expressing Nef-YN and Nef-YC were stained with

antibodies to the TGN marker, TGN-46, and a secondary antibody conjugated to Texas red. The merged image shows co-localization of the perinuclear BiFC signal and the TGN marker (arrows). Scale bar, 5 μ m.

Figure 4.

Efficiency of Nef BiFC. (a) Nef-YN and Nef-YC were co-expressed in 293T cells. Forty-eight hours later, cells were fixed and stained with a monoclonal antibody to the Nef protein and a secondary antibody Texas-red conjugate. A strong BiFC signal (Nef-BiFC; green) was observed in virtually all cells found to express Nef by immunofluorescence (Nef-IF; red). Both the Nef BiFC and IF signals exhibit the same subcellular localization pattern (Merge). Scale bar, 25 μ m. (b) Pixel intensities of individual cells exhibiting both Nef expression by immunofluorescence and dimerization by BiFC were quantified using ImageJ (n=100). A linear correlation exists between the intensity of BiFC and the level of Nef expression (correlation coefficient of 0.72 as determined using Microsoft Excel).

Figure 5.

Mapping Residues Essential for Nef Dimerization by BiFC. The Nef dimerization interface was mutated by replacing all four hydrophobic core residues (I109, L112, Y115, F121) with aspartate (4D mutant), by substitution of Tyr-115 with aspartate (Y115D), or by replacing Asp-123 with either asparagine (D123N) or valine (D123V). 293T cells were transfected with wild-type (WT) or mutant Nef-YN and Nef-YC expression vectors and counterstained with anti-Nef antibodies and visualized with secondary antibodies conjugated to Texas-red. Representative images are shown for the BiFC signal (Nef-BiFC; green) and Nef immunofluorescence (Nef-IF; red) for the same field of cells. Scale bar, 100 μ m.

Figure 6.

Nef Dimerization is Required for CD4 Downregulation. (a) SupT1 cells were infected with a wild-type Nef retrovirus (Nef-WT) or with retroviruses carrying the BiFC-negative Nef mutants 4D and D123N. Cell populations were selected with G418, and cell surface levels of CD4 were assessed by flow cytometry relative to uninfected parental SupT1 cells as described under Experimental Procedures. CD4 staining in the parental cell population is shown in the upper left panel, and is overlaid in green on the analyses of the cells expressing wild-type and mutant forms of Nef. (b) Cell-surface expression of CD4 was determined by flow cytometry in SupT1 cells expressing myristoylation-defective Nef (G2A), wild-type Nef (WT), and the Nef dimerization interface mutants Y115D, 4D, D123A, D123N, D123V, and 2RE. Data are

expressed as mean percent downregulation relative to cell-surface CD4 levels observed in uninfected control cells ($\pm$ S.D.; n=3). (c) Expression of wild-type and mutant forms of Nef was confirmed in the SupT1 cell populations by immunoblotting of cell lysates with anti-Nef antibodies. Actin blots were also performed as loading controls.

Figure 7. Mutations in the Dimerization Interface Do Not Disturb the Three-Dimensional Fold of Nef Required for SH3-binding Activity. (a) Crystal structure of a high-affinity Nef:SH3 complex where the SH3 domain is shown in green and the Nef core monomer in blue (**PDB:1EFN**). Key Nef residues involved in this interaction are indicated and include Nef PxxP helix and residues Phe 90, Trp 113 and Tyr 120 that form a hydrophobic pocket in the folded Nef core. This Nef pocket interacts with SH3 RT loop residue Ile-96 and is essential for high-affinity docking of the SH3 domain. (b) SH3-binding activity of wild-type and dimerization-defective Nef mutants revealed an equivalent recovery of the GST-SH3 fusion proteins from each reaction. Nef proteins tested in this experiment included wild-type (WT), D123A (DA), D123N (DN), D123V (DV), and 4D (I109D/L112D/Y115D/F121D).

Figure 8. Nef Dimerization is Required for HIV Replication

Upper panel: U87MG/CD4/CXCR4 target cells were infected with equivalent titers (250 pg/ml) of HIV_{NL4-3/SF2} that expresses wild-type Nef (WT), fails to express Nef (ΔNef), or expresses the dimerization interface mutants L112A, Y115D, 4D, D123A, D123N, D123V and 2RE. Viral replication was monitored by determination of HIV p24 levels in culture lysates by ELISA 4 days later. This experiment was repeated five times, and the data are shown as percent of wild-type p24 levels $\pm$ S.D. *Lower panel:* Expression of wild-type and mutant forms of Nef from each of the proviruses was confirmed by immunoblotting transfected 293T producer cell lysates with anti-Nef antibodies. Note that the epitope for the antibody partially overlaps with the mutated region in Nef-4D, accounting for the reduced immunoreactivity of this mutant compared to the other Nef proteins.

Table 1

BiFC analysis of Nef dimerization interface mutants. 293T cells were co-transfected with paired BiFC expression vectors for wild-type Nef as well as each of the dimerization interface mutants shown. Forty-eight hours later, cells were immunostained for Nef protein expression and the relative intensities of the BiFC and immunofluorescence signals were quantified using MetaMorph software. Each Nef BiFC signal was then normalized to the Nef protein level as determined by immunofluorescent staining. The percent decrease in BiFC relative to wild-type Nef was then calculated by dividing the normalized BiFC signal for each mutant by the wild-type value, subtracting the result from 1.0, and multiplying by 100. This experiment was repeated in quadruplicate with comparable results in each case. While all mutants decreased Nef-BiFC, the most dramatic decreases were consistently observed with the 4D and D123N mutants (bold).

Nef Mutant	% Decrease in BiFC Relative to WT
L112A	52.8
F121A	33.0
L112D	47.5
Y115D	59.1
L112D/Y115D (DD)	86.7
I109D/L112D/Y115D/F121D (4D)	99.9
D123A	69.6
D123N	97.1
D123V	52.9
R105E	49.9
R105E/R106E (2RE)	71.1
G2A (myristoylation defective)	0.0
2PA (SH3-binding motif)	0.0