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Short Communication

Nef protein of human immunodeficiency virus type 1 binds its own myristoylated N-terminus

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Abstract

HIV-1 Nef is a small protein (approx. 25 kDa) that is posttranslationally modified by myristoylation. To explain its complex activities, a 'Nef-cycle' is discussed, which postulates different molecular conformations of Nef. Using recombinant full-length non-myristoylated Nef and synthetic peptides, we demonstrate by fluorescence titration experiments that a peptide representing the myristoylated N-terminus of Nef is specifically bound by Nef. A non-myristoylated N-terminal fragment of Nef or a myristoylated control peptide does not bind to Nef. These results are the first direct experimental evidence of the existence of a myristate-binding pocket in Nef, a prerequisite of the postulated 'closed' Nef conformation.

Keywords: fluorescence titration; peptide binding; protein conformation.

Human immunodeficiency virus type 1 (HIV-1) Nef is a small protein of approximately 25 kDa that is essential for high-titer viral replication and pathogenesis of acquired immunodeficiency syndrome (AIDS). Nef is produced in the earliest stage of viral gene expression and is a component of viral particles. It is posttranslationally modified by myristoylation of the N-terminal glycine (residue 2 in the SwissProt numbering scheme) and by phosphorylation of at least one serine residue. Nef interacts with a multitude of host cellular proteins. Its multiple functions, e.g., its interaction with various signal transduction proteins such as p21-activated kinase and cytoplasmic protein tyrosine kinases (Renkema and Saksela, 2000) and Nef-mediated down-modulation of CD4, MHC class I, CD28, CXR4 and CD8$\alpha\beta$ (Mangasarian et al., 1999; Bell et al., 2001; Swigut et al., 2001; Hrecka et al., 2005; Stove et al., 2005), involve cytoplasmic and membrane-bound stages. The loss of a special Nef function, namely the Nef-mediated suppression of T-cell activation, is held to be responsible for the progression to AIDS in HIV-1-infected humans, which is contrary to the non-pathogenic processing of most SIV infections (Schindler et al., 2006). Very recently, interference in the maturation of stimulatory T-cell contacts by modulation of N-WASP activity through Nef was described (Haller et al., 2006). To explain the complex activities of the viral protein, a 'Nef-cycle' is discussed, which postulates different molecular conformations of Nef (Arold and Baur, 2001; Dennis et al., 2005). It is hypothesized that Nef undergoes a substantial conformational change from an 'open' to a 'closed' form, whereby the myristate group is thought to be sequestered into a binding pocket. Myristoylated Nef may then switch to the open conformation by association of the N-terminal region with membranes. Experimental proof of such a myristate-binding pocket in Nef, however, is still lacking. Structural studies by nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography to address the postulated binding pocket are severely hampered by the extremely high aggregation tendency of myristoylated Nef (myr-Nef) at concentrations necessary for these experiments (Dennis et al., 2005). Therefore, we used another approach to investigate the potential myristate-binding pocket of Nef. Although it was reported that myristate is not bound by Nef (Geyer et al., 2001), it may well be that myristate is bound by Nef if covalently linked to the N-terminal part of Nef. To address this question, we used unmodified recombinant full-length Nef protein starting with residue glycine 2 (Nef_{2-210}) and studied its binding to a synthetic myristoylated Nef_{2-7} peptide (myr-Nef_{2-7}), an unmodified Nef_{2-7} peptide, and a myristoylated control peptide (myr-Pep) (Figure 1).

Nef_{2-210} was expressed in *E. coli* BL21(DE3) RIL cells harboring plasmid pUbi-Nef_{2-210} carrying the Nef gene

myr-Nef_{2-7}:	Myristoyl- G G K W S K(FITC)
Nef_{2-7}:	G G K W S K(FITC)
myr-Pep:	Myristoyl- Q D S K(FITC)

Figure 1 Overview of the FITC peptides used for Nef binding studies.
Amino acid sequences are given using the one letter-code for the peptides named on the left. The myristoyl modification, as well as the position of the fluorescein isothiocyanate label (FITC), is indicated. All peptides were purchased from JPT Peptide Technologies GmbH (Berlin, Germany), with their C-termini blocked by amidation.

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from the HIV-1 isolate SF2 (SwissProt accession no. P03407) as a poly-histidine-tagged (His-tagged), ubiquitin-fused construct in a pTKK19xb/ub vector (Kohno et al., 1998). Bacteria were grown aerobically at 37°C in Luria broth containing kanamycin (100 μg/ml) and chloramphenicol (34 μg/ml). Cells at $OD_{600\ nm}$ of ~0.8 were induced with isopropyl β-D-thiogalactopyranoside (IPTG; final concentration 0.2 mM) for 3 h, harvested by centrifugation and sonified in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 8.0, supplemented with lysozyme and protease inhibitors). The extract was clarified by centrifugation and applied to a Ni-NTA metal affinity column (Qiagen, Hilden, Germany). After extensive washing (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole), the protein was eluted by increasing the imidazole concentration stepwise from 10 to 250 mM. Fractions containing pure fusion protein (usually fractions with 100 mM imidazole), as determined by denaturing SDS-PAGE, were pooled and dialyzed against cleavage buffer and subsequently cleaved using His-tagged yeast ubiquitin hydrolase (Kohno et al., 1998). Nef_{2-210} was separated from His-tagged ubiquitin, uncleaved fusion protein and His-tagged yeast ubiquitin hydrolase by a second Ni-NTA metal affinity column equilibrated with cleavage buffer, with Nef_{2-210}, notably without any artificial additional amino acid residue, located in the flow-through (Figure 2). To assure protein solubility, fractions containing pure Nef_{2-210} were directly supplemented with NaCl to a final concentration of 300 mM and concentrated using an Amicon flow cell under N_2 atmosphere. All buffers used in this study were degassed and supplemented with 14 mM β-mercaptoethanol. The identity of the Nef_{2-210} protein was confirmed by in-gel tryptic digestion of the electrophoretically separated protein and mass spectrometric peptide mapping. The final yield of Nef_{2-210} was 8 mg/l.

Fluorescence titrations were carried out by measuring the fluorescence of a peptide comprising residues 2–7 of Nef (GGKWSK), with the N-terminal glycine being myristoylated and the C-terminal lysine carrying a fluorescein isothiocyanate (FITC) residue covalently linked to the ε-amino group (myr-Nef_{2-7}) in the presence of increasing concentrations of Nef_{2-210} (Figure 3A). Titrations were performed as previously described, and no binding of Nef to the FITC label was found (Preusser et al., 2001). Assuming a simple bimolecular reaction between Nef and the peptides, analysis by non-linear curve fitting yielded a K_d value of 8.7 μM with a standard deviation of 2.0. No binding was observed for the unmyristoylated Nef_{2-7} peptide (Figure 3A). As a further control, an arbitrarily selected myristoylated control peptide (myr-Pep) was also titrated with Nef (Figure 3A). Owing to the extremely weak binding indicated by the linear decrease in Nef-concentration-dependent fluorescence, a dissociation constant could not be determined, but is clearly greater than 50 μM. Linear regression analysis of the Scatchard plot of myr-Nef_{2-7} titration with Nef confirmed the dissociation constant (-1/K_d, slope). In addition, the x-axis intercept of the Scatchard plot yielded the number of Nef binding sites per myr-Nef_{2-7} peptide (Figure 3B). The value is slightly greater than 1, probably due to partial oligomerization of Nef leading to a lower apparent concentration of Nef monomers. Such a concentration-dependent oligomerization of non-myristoylated Nef is well known and is fully reversible (Arold et al., 2000).

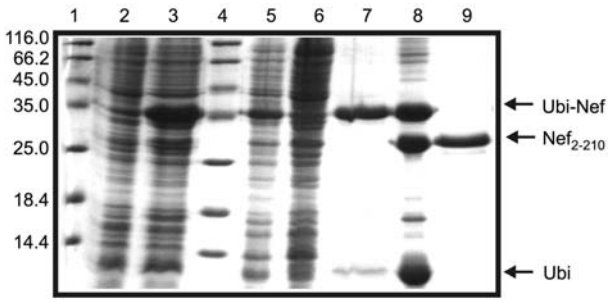

Figure 2 Expression and purification of recombinant Nef_{2-210} from HIV-1 isolate SF2.
Expression of pUbi-Nef in *E. coli* (DE3) RIL before (lane 1) and after (lane 2) induction with 0.2 mM IPTG. Purification of Nef_{2-210}: soluble fraction of the cell lysate (lane 5); flow-through fraction (lane 6) and pooled His-tagged ubiquitin-Nef fusion protein (Ubi-Nef) containing fractions (lane 7) after Ni^{2+}-chelating affinity chromatography; cleavage (lane 8) of Ubi-Nef with yeast ubiquitin hydrolase and second Ni^{2+}-chelating affinity chromatography (lane 9); Nef_{2-210} is located in the flow-through fraction; M, molecular mass markers (in kDa). Ubi-Nef, Nef(2–210) and His-tagged ubiquitin (Ubi) are marked by arrows.

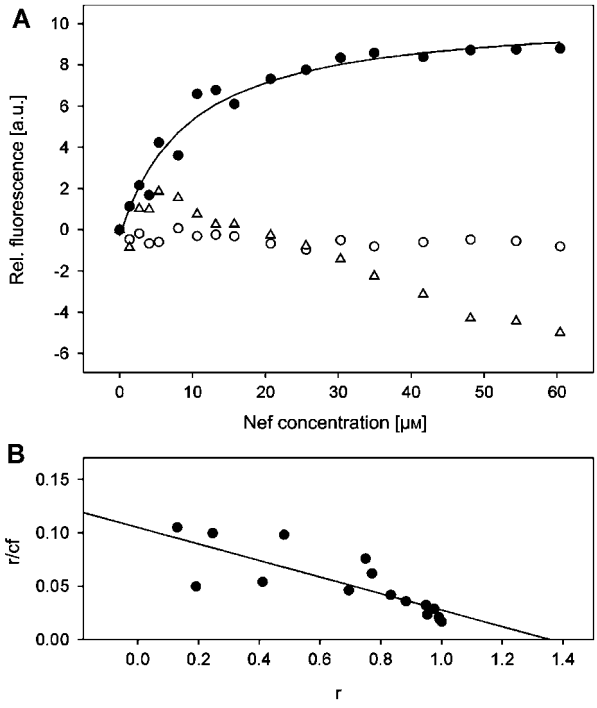

Figure 3 Fluorescence titration studies.
(A) Fluorescence of 1.0 μM myr-Nef_{2-7} (●), 1.0 μM Nef_{2-7} (○) and 1.0 μM control peptide (myr-Pep) (Δ) as a function of Nef concentration. Each titration was repeated twice and yielded fully reproducible results. The non-linear fit obtained for Nef binding to myr-Nef_{2-7} is shown by a solid line. (B) Scatchard plot of the data points shown in (A) for binding of HIV-1 Nef to myr-Nef_{2-7} (●) peptide. The x-axis (r) corresponds to the ratio of bound Nef to total myr-Nef_{2-7} peptide concentration, and r/cf on the y-axis is the ratio of r and unbound Nef. Linear regression analysis (solid line) yielded the dissociation constant (-1/K_d, slope), and the number of Nef binding sites per myr-Nef_{2-7} peptide molecule (x-axis intercept).

The titration experiments in our study clearly show that full-length Nef is able to bind a peptide comprising its own N-terminus, if and only if it is N-terminally myristoylated. This is the first direct experimental evidence of the existence of such a binding site on Nef. This in turn is a prerequisite for the postulated 'closed' form of Nef, which is defined by an interaction between the myristoylated N-terminus and the core. The closed conformation of Nef helps to explain why modified Nef is approximately 50% soluble when being expressed recombinantly (Dennis et al., 2005), and remains cytosolic in HeLa cells to approximately 75% (Bentham et al., 2006). The binding site of Nef for its myristoylated N-terminus nicely explains the drastically increased aggregation tendency of myristoylated Nef compared to unmodified Nef at higher protein concentrations. Sequestration of the myristoylated N-terminus by intramolecular contacts has been suggested as the mechanism that renders modified Nef soluble and monomeric at low protein concentrations (Dennis et al., 2005). We assume that at higher protein concentrations, at least *in vitro*, intermolecular interactions between myristoylated Nef molecules may occur, potentially leading to high-molecular-weight assemblies. Beside the 'open' conformation of modified Nef discussed, in which largely hydrophobic surfaces are exposed, this might result in insolubility and aggregation, and might be one reason for the many different Nef species described, e.g., during dynamic light scattering experiments (Dennis et al., 2005).

It is hypothesized that contact with the cellular membrane could trigger a conformational change in Nef, thus relieving the interaction between the myristoylated N-terminus and the core, which subsequently might expose various interaction motifs of Nef to other factors. This regulatory step might be an interesting target for novel therapeutic approaches to HIV.

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