A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4

Michael Greenberg*, Louis DeTulleo†‡, Iris Rapoport‡, Jacek Skowronski* and Tomas Kirchhausen‡

Nef, a ~200 residue multifunctional regulatory protein of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), interacts with components of host cell signal transduction and clathrin-dependent protein sorting pathways. The downregulation of surface CD4 molecules and major histocompatibility complex (MHC) class I antigens by Nef is believed to be important in AIDS pathogenesis [1–7]. Nef contains a globular core domain and two disordered segments – a myristylated arm at the amino terminus and a carboxy-terminal loop projecting from the globular core [8,9]. Here, we aimed to determine the sorting signals in HIV-1 Nef that were responsible for its involvement in the clathrin-mediated pathway. We found that a sequence in the carboxy-terminal disordered loop of Nef is essential for downregulation of CD4. This sequence resembles the dileucine motif, one of two well-characterized sorting signals that target membrane proteins to clathrin-coated vesicles. The dileucine-motif-containing segment of Nef bound directly and specifically to the β-adaptin subunit of the clathrin adaptor complexes AP-1 and AP-2, which are responsible for recruiting sorted proteins into coated pits. Unlike wild-type Nef, a mutant form of Nef that lacked the dileucine motif did not localize to clathrin-coated pits and did not downregulate CD4 expression, although it could downregulate MHC class I surface expression. Thus, the dileucine motif in HIV-1 is required for CD4 downregulation and for interaction with clathrin adaptor complexes.

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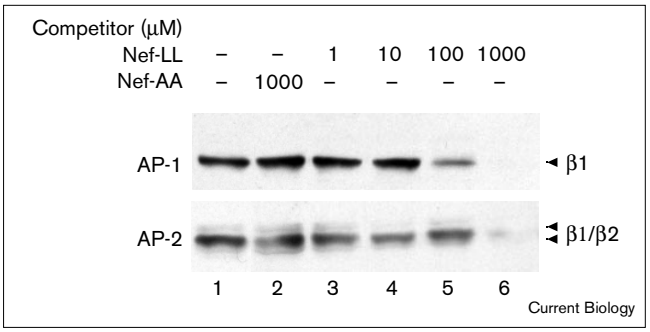
Results and discussion

Mutations in short sequences in the two disordered loops of HIV-1 Nef prevent localization of Nef to coated pits and CD4 downregulation, but these sequences do not correspond to canonical sorting signals [10]. It has been claimed that Nef interacts with adaptor proteins through a tyrosine-based motif of the type YppØ (where Y is tyrosine, p is a polar residue and Ø is a bulky hydrophobic amino acid) in the amino-terminal arm, but the two YppØ motifs identified in that study (which was performed on SIV mac239 Nef) are absent in Nef proteins from HIV-1 strains (although similar tyrosine-based sequences are present in HIV-2 Nef) [11]. Therefore, this motif is probably not the essential targeting motif directing Nef traffic, although it may have an auxiliary role.

As many of the proteins that traffic via clathrin-dependent pathways contain either dileucine or YppØ motifs [12], we examined the primary structures of HIV-1 Nef for candidate sorting signals. Although there were no conserved YppØ motifs, a conserved dileucine sequence surrounded by polar residues was identified in an otherwise variable region of the protein located in the carboxy-terminal disordered loop (see Supplementary material published with this paper on the internet). A similar sequence (LM or LV) is found at the corresponding position in HIV-2 and SIV Nef. The dileucine sorting signal not only requires a leucine doublet, but also requires polar surrounding sequences [13], presumably causing the dileucine sequence to be exposed on the surface of the protein. A glutamic acid residue is always present at position –4 relative to the first leucine of the leucine doublet. This sequence in Nef thus has all the characteristics of a dileucine sorting signal. We therefore investigated whether a peptide containing this sequence could interact directly with adaptor protein complexes.

To determine whether the dileucine-containing region contains an active sorting dileucine motif, we used a crosslinking protocol in which peptides and adaptor proteins were first frozen, and then subjected to crosslinking in the frozen state, a condition that does not affect the specificity of interactions between the adaptor protein and peptide [13,14]. Using this method, peptides bearing a dileucine sorting signal were found to bind specifically to the β1-adaptin subunit of AP-1 [13]. Those experiments were performed in the presence of detergent, however, and the interactions with the β-adaptin subunits of AP-2 were too weak to detect under such conditions. Repeating the experiments in the absence of detergent led to the interaction with AP-1 becoming stronger and the interactions with the β1-adaptin and β2-adaptin subunits of AP-2 being readily detectable.

Figure 1



Specific interaction of the dileucine-based motif of HIV-1 NA7 Nef with the β-adaptin chains of clathrin adaptors. Analysis of the crosslinking of the UV-photoactive peptide *CD3γ-LL (0.2 μM) with adaptor proteins in the absence (lane 1) and presence of the competing peptides EEANTGENNSAAHPMS (Nef-AA; lane 2) and EEANTGENNSLLHPMS (Nef-LL; lanes 3–6) derived from the disordered loop of HIV-1 NA7 Nef. *CD3γ-LL is known to associate specifically with the β-adaptin subunits of AP-1 and AP-2 [13]. The data were obtained using ~0.2 mg/ml AP-1 (upper panel) or AP-2 (lower panel) purified from calf brain coated vesicles. AP-1 contains β1-adaptin chains, whereas AP-2 has mostly β2-adaptin chains and some β1-adaptin chains [21].

The specificity of the interaction can be examined by using the test peptides as competitors for a biotinylated dileucine-motif-containing peptide derived from the γ subunit of the T-cell receptor that also contains the UV-activatable crosslinker benzoyl phenylalanine (*CD3γ-LL; Figure 1, lane 1). The Nef-derived peptide EEANTGENNSLLHPMS from HIV-1 NA7 (Nef-LL) [4], like other peptides containing active dileucine motifs [13], specifically blocked the interaction between *CD3γ-LL and the ~100 kDa β-adaptin subunits of AP-1 and AP-2 (Figure 1, lanes 3–6). In contrast, a mutant Nef peptide in which the two leucine residues ($L_{164}L_{165}$) were replaced by alanine residues (Nef-AA) did not bind to β1-adaptin or β2-adaptin (Figure 1, lane 2). The apparent inhibition constants (IC_{50}s), corresponding to the amount of peptide required to inhibit the crosslinking reaction by 50%, probably indicate that the interactions with AP-2 are weaker than with AP-1; the absolute IC_{50} values cannot be determined with confidence because the concentrations of peptide and adaptor proteins in areas of the frozen solution are higher relative to those in the liquid solution [13]. Thus, the clathrin adaptor proteins specifically recognize the dileucine motif found in Nef. This result is consistent with the association between GST–Nef and AP-1 detected *in vitro* [15].

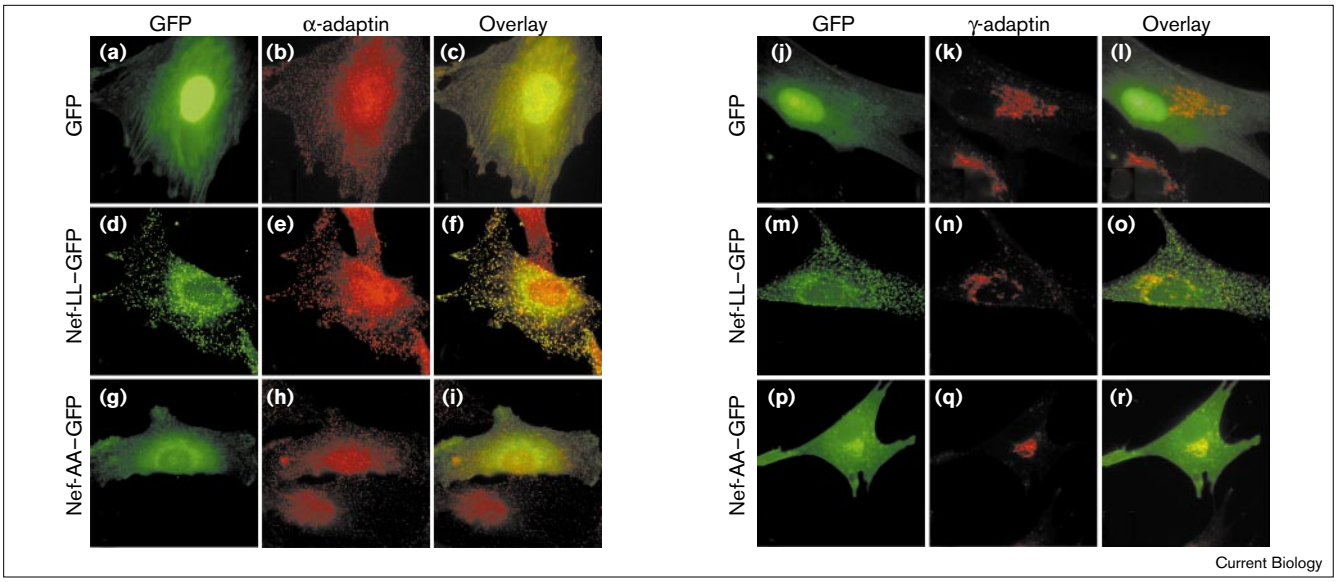
We next asked whether Nef mutants that lack the dileucine motif retain the ability to localize with AP-1 and AP-2 adaptors in clathrin-coated pits. As previously shown [10], the HIV-1 NA7 Nef protein tagged with green fluorescent protein (GFP), Nef-LL–GFP, co-localized with adaptors in clathrin-coated pits at the plasma membrane (Figure 2d–f) and in the trans-Golgi network (TGN; Figure 2m–o). We constructed a mutant Nef–GFP fusion protein in which the two leucine residues were replaced with alanines (Nef-AA–GFP) and expressed it in rat REF52 and in human IMR90 fibroblasts by transient transfection. The Nef-AA–GFP mutant retains the myristylation sequence, and should be targeted normally to intracellular membranes. This mutant failed to localize with AP-2 adaptors (α-adaptin staining) at the cell surface (Figure 2g–i). We can rule out the possibility that the failure to localize with AP-2 is due to gross misfolding of the Nef-AA–GFP protein because this mutant has the same ability to downregulate MHC class I as the wild-type Nef-LL–GFP fusion protein (see below). The perinuclear localization for Nef-LL–GFP (Figure 2m–o) and Nef-AA–GFP (Figure 2p–r) was similar in appearance and overlapped the tight distribution of AP-1 adaptors (γ-adaptin staining) at the TGN. It should be noted, however, that the resolution of the images does not allow us to establish with certainty whether the overlap of the Nef–GFP proteins with AP-1 is specific for coated pits at the TGN. We conclude that the dileucine motif is essential for Nef targeting to clathrin-coated pits containing AP-2 at the plasma membrane.

To determine whether the dileucine motif is important for Nef's biological function, we examined whether the Nef-AA mutant would still downregulate CD4. As previously described [16], $CD4^+$ Jurkat T cells transiently transfected with wild-type HIV-1 NA7 Nef downregulated CD4 from the cell surface (Figure 3b,e); this effect was also observed using HIV-1 NL4-3 Nef fused to GFP (data not shown) [10]. The Nef-AA mutant derived from either of these strains had no detectable effect on surface CD4 levels, however (Figure 3c,f and data not shown). As indicated by the comparative dose–response experiments illustrated in Figure 3g, Nef-LL facilitated the downregulation of CD4 over a wide range of Nef expression levels whereas the Nef-AA mutant did not. However, the LL→AA mutation did not affect the ability of Nef to downregulate MHC class I (compare Figure 3b,c with Figure 3e,f). Thus, the dileucine signal is required both for Nef targeting to coated pits and for CD4 downregulation, but not for MHC class I downregulation.

Our results show that the interaction of Nef with clathrin adaptor complexes and the Nef-stimulated downregulation of CD4 both depend on a dileucine motif in the carboxy-terminal disordered loop of Nef. The Nef internalization motif identified in this study is highly conserved in HIV-1, but it is not the only sequence involved in Nef traffic and CD4 internalization [10,17]. The dileucine motif is present in a modified form (LM or LV) in HIV-2 and SIV (see Supplementary material). Whether these amino acids also influence Nef's effect on CD4 is now under investigation.

Nef also interacts with the 56 kDa subunit of the vacuolar membrane ATPase complex (V-ATPase) [17]. The region

Figure 2

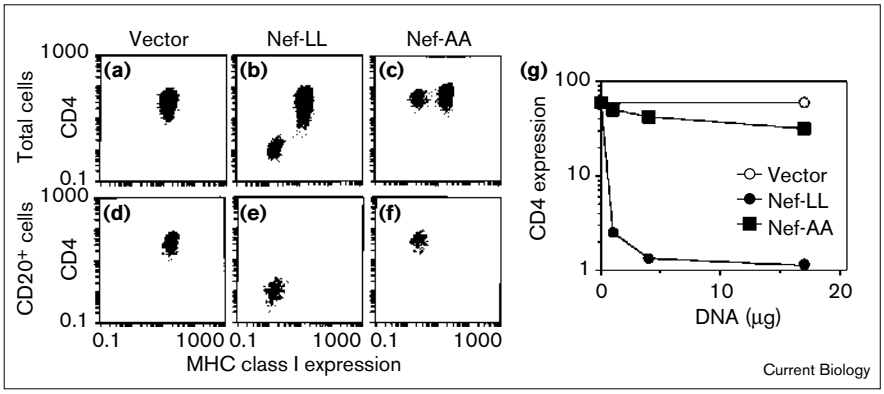


The dileucine motif of Nef is required for the colocalization of Nef and clathrin adaptor complexes. **(a–i)** REF52 rat fibroblasts or **(j–r)** IMR90 human fibroblasts transiently expressing (a–c, j–l) GFP, (d–f, m–o) Nef-LL–GFP, or (g–i, p–r) Nef-AA–GFP derived from HIV-1 NA7 Nef were fixed and processed for immunofluorescence. The localization of clathrin-coated pits containing AP-2 was determined in REF52 cells using a rabbit polyclonal antibody specific for the α-adaptin subunits of clathrin adaptor complex AP-2. The localization of clathrin-coated pits containing AP-1 was determined in IMR90 cells using the mouse monoclonal antibody 100/3 specific for the γ-adaptin subunit of the non-rodent clathrin adaptor AP-1, and Texas Red conjugated goat anti-rabbit or goat anti-mouse IgG antibody (red). GFP products were localized by direct fluorescence (green). The overlay of GFP and α-adaptin images shows colocalization of Nef-LL–GFP but not of Nef-AA–GFP with AP-2 in coated pits at the plasma membrane.

required for this interaction was mapped to $E_{178}D_{179}$ in HIV-1 SF2 Nef (equivalent to $D_{174}D_{175}$ in HIV-1 NL4-3 Nef) at the carboxy-terminal boundary between the disordered loop and the core domain. A $D_{174}D_{175}$→AA HIV-1 NA7 Nef mutant did not localize to clathrin-coated pits [18] or downregulate CD4 [16,17]. The counterintuitive finding that both the $D_{174}D_{175}$→AA and the $L_{164}L_{165}$→AA Nef mutants affect CD4 but not MHC class I downregulation indicates that the simple model in which Nef interacts (directly or indirectly) with CD4 and MHC class I and

Figure 3

The effect of the Nef-AA mutant on the expression of CD4 and MHC class I on the cell surface. **(a–f)** Flow cytometry analysis. CD4+ Jurkat T cells were transiently transfected with 18 μg (a,d) control empty expression vector DNA (vector) or 18 μg DNA encoding (b,e) wild-type HIV-1 NA7 Nef (Nef-LL) or (c,f) mutant Nef (Nef-AA), together with 2 μg plasmid DNA encoding the surface marker CD20 for the identification of transfected cells. Flow cytometry analysis was performed 2 days after transfection on (a–c) the total population of cells or (d–f) the cells expressing the CD20 transfection marker. CD4, MHC class I, and CD20 were detected simultaneously by three-color flow cytometry. The surface levels of MHC class I (x-axis) and CD4 (y-axis) were detected with G46-2.6 monoclonal antibody labeled with fluorescein and with Leu3 monoclonal antibody labeled with phycoerythrin, respectively. PerCP-conjugated



Leu16 monoclonal antibody was used to detect CD20+ cells. **(g)** Dose–response analysis for the effect of the Nef-AA mutant on CD4 surface expression. CD4+ Jurkat T cells were transfected with increasing amounts of plasmid DNA encoding wild-type HIV NA7 Nef (Nef-LL), mutant Nef (Nef-AA) and empty vector as described for (a–f). Surface expression of CD4 (y-axis) is shown as the peak channel number of the CD4 signal.

recruits them into clathrin-coated pits is not likely to be correct. Clearly MHC class I downregulation occurs via a separate mechanism that is not affected by either V-ATPase binding or interaction with adaptor proteins [17,18]. It is also surprising that, although both the $D_{174}D_{175}$ residues and the dileucine motif are necessary for the localization of Nef to coated pits, neither of these two elements is alone sufficient for localization.

The model that emerges from these studies is a complex one: Nef must contain at least four functional regions required for its effects on membrane traffic, one responsible for interacting with V-ATPase [17], the second containing a motif for binding to adaptor protein complexes (this study), the third providing a direct or indirect interaction with CD4 [10,19,20] and the fourth required for MHC class I downregulation [3,6,15,18]. From our results and those of Lu *et al.* [17], it would appear that Nef requires both the V-ATPase interaction domain and the adaptor-protein-binding motif to localize to coated pits and induce CD4 downregulation. These interactions may therefore be suitable targets for drug design.

Material and methods

AP-1 and AP-2 adaptor complexes were purified from calf brain coated vesicles [13,14] and suspended in AP buffer (100 mM MES, 1 mM EDTA, 150 mM NaCl, 0.5 mM DTT, pH 7.0). The UV-photoactive crosslinking reaction and the assay to determine the specific interaction between dileucine-motif-containing peptides and AP subunits has been described previously [13,14]. Briefly, purified adaptors (~0.2 mg/ml in 18 µl AP buffer) were mixed with 0.2 µM photoactive peptide *CD3γ-LL (biotin-KQS (benzoylphenylalanine)ApSDKQTLLPN, where pS denotes phosphoserine) in the presence or absence of non-modified competing peptide in a final volume of 22 µl. The mixtures were first frozen by placing them on top of pulverized dry ice and then exposed to UV light for 3 min. The identity of the AP subunit crosslinked to the biotinylated *CD3γ-LL was established by SDS–PAGE, followed by electrotransfer to nitrocellulose, blotting with streptavidin–HRP and detection by enhanced chemiluminescence (Amersham) on X-ray film. The interaction of *CD3γ-LL is specific, as crosslinking to the β-adaptin chains is inhibited by co-incubation with non-modified peptides containing the dileucine motif of CD3γ (RQSRApSDKQTLLPN), CD4 (RMpSQIKRLLSEK), Glut4 (ISAAFRRTpSLLEQEVKPSTEL) and M6PR (VSFHDDSDEDLLHI) [13]. Various amounts of the synthetic peptides EEANKGENTSLLHPMS (for Nef-LL) and EEANKGENTSAAHPMS (for Nef-AA) of strain NA7 were monitored for their ability to prevent the specific crosslinking reaction between *CD3γ-LL and the β1/β2 subunits of AP-1 and AP-2. Plasmid construction, DNA transfections, flow cytometry and fluorescent light microscopy analysis were performed as described previously [10,16,18].

Supplementary material

A detailed background section and a figure showing the conservation of the dileucine motif in Nef proteins is published with this paper on the internet.

Acknowledgements

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References

1. Schwartz O, Dautry-Varsat A, Goud B, Marechal V, Subtil A, Heard JM, Danos O: **Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes.** *J Virol* 1995, **69:**528-533.
2. Garcia JV, Miller AD: **Serine phosphorylation-independent downregulation of cell-surface CD4 by nef.** *Nature* 1991, **350:**508-511.
3. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D: **HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.** *Nature* 1998, **391:**397-401.
4. Mariani R, Skowronski J: **CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals.** *Proc Natl Acad Sci USA* 1993, **90:**5549-5553.
5. Mariani R, Kirchhoff F, Greenough TC, Sullivan JL, Desrosiers RC, Skowronski J: **High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection.** *J Virol* 1996, **70:**7752-7764.
6. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM: **Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein.** *Nat Med* 1996, **2:**338-342.
7. Anderson S, Shugars DC, Swanstrom R, Garcia JV: **Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells.** *J Virol* 1993, **67:**4923-4931.
8. Grzesiek S, Bax A, Clore GM, Gronenborn AM, Hu J-S, Kaufman J, *et al.*: **The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase.** *Nat Struct Biol* 1996, **3:**340-345.
9. Lee CH, Saksela K, Mirza UA, Chait BT, Kuriyan J: **Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain.** *Cell* 1996, **85:**931-942.
10. Greenberg ME, Bronson S, Lock M, Neumann M, Pavlakis GN, Skowronski J: **Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation.** *EMBO J* 1997, **16:**6964-6976.
11. Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, Trono D: **Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the µ chain of adaptor complexes.** *EMBO J* 1998, **17:**2472-2481.
12. Kirchhausen T, Bonifacino JS, Riezman H: **Linking cargo to vesicle formation — receptor tail interactions with coat proteins.** *Curr Opin Cell Biol* 1997, **9:**488-495.
13. Rapoport I, Chen YC, Cupers P, Shoelson SE, Kirchhausen T: **Dileucine-based sorting signals bind to the β chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif-binding site.** *EMBO J* 1998, **17:**2148-2155.
14. Rapoport I, Miyazaki M, Boll W, Duckworth B, Cantley LC, Shoelson S, Kirchhausen T: **Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes.** *EMBO J* 1997, **16:**2240-2250.
15. Le Gall S, Erdtmann L, Benichou S, Berlioz-Torrent C, Liu L, Benarous R, *et al.*: **Nef interacts with the µ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules.** *Immunity* 1998, **8:**483-495.
16. Iafrate AJ, Bronson S, Skowronski J: **Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling.** *EMBO J* 1997, **16:**673-684.
17. Lu X, Yu H, Liu SH, Brodsky FM, Peterlin BM: **Interactions between HIV-1 Nef and vacuolar ATPase facilitate the internalization of CD4.** *Immunity* 1998, **8:**647-656.
18. Greenberg ME, Iafrate AJ, Skowronski J: **The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes.** *EMBO J* 1998, **17:**2777-2789.
19. Grzesiek S, Stahl SJ, Wingfield PT, Bax A: **The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR.** *Biochemistry* 1996, **35:**10256-10261.
20. Mangasarian A, Foti M, Aiken C, Chin D, Carpentier JL, Trono D: **The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane.** *Immunity* 1997, **6:**67-77.
21. Clairmont KB, Boll W, Ericsson M, Kirchhausen T: **A role for the hinge/ear domain of the beta chains in the incorporation of AP complexes into clathrin-coated pits and coated vesicles.** *Cell Mol Life Sci* 1997, **53:**611-619.

Supplementary material

A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for down-regulation of CD4

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Supplementary background

Nef is a multifunctional regulatory protein of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) which interacts with host cell signal transduction and protein sorting machinery. Nef appears to be an important determinant of the clinical outcome in people infected with HIV-1 [S1,S2] and in SIV infected rhesus macaques [S3]. Downregulation of surface CD4 molecules and of major histocompatibility complex (MHC) class I are conserved functions of Nef that are believed to have roles in AIDS pathogenesis [S4–S10].

The normal route for CD4 traffic involves entrapment into clathrin-coated pits and endocytosis, followed by re-sorting and return to the plasma membrane [S11]. Three HIV-1 gene products, Env, Vpu and Nef downregulate expression of cell-surface CD4, and Nef is a major component of this downregulation [S12]. Nef accelerates CD4 internalization, without accelerating recycling to the cell surface [S13]. As well as causing downregulation of CD4, Nef also causes the surface downregulation of MHC class I complexes [S9]. Nef localizes to the internal leaflet of the plasma membrane [S14], and it is therefore possible that it may interact directly or indirectly with the cytoplasmic tails of CD4 or MHC class I.

Nef is a myristylated cytoplasmic protein of ~200 residues. It can be divided into three regions, a globular core domain and two disordered segments, one a myristylated arm at the amino terminus, and the other a loop projecting from the globular core (Figure S1a) [S15,S16]. Nef is the first non-transmembrane protein located in the internal side of the plasma membrane that is known to be sorted in the clathrin-dependent pathway, being able to localize in clathrin-coated pits even in the absence of other viral proteins and in the absence of CD4 [S14]. Some evidence has been presented to indicate that CD4 and Nef may directly interact; for example, NMR studies using peptides from the cytoplasmic tail of CD4 appear to show a weak association between the membrane-proximal portion of the CD4 tail and the Nef protein [S17]. Therefore, one possible model for CD4 downregulation is that Nef binds directly to CD4 and drags it into clathrin-coated pits [S14,S18].

Site-directed mutagenesis of cellular proteins that traffic through the clathrin-dependent pathway has identified several motifs including the dileucine (LL) and tyrosine-based (YppØ) sorting signals (where L is leucine, Y is tyrosine, p is a polar residue and Ø is a bulky hydrophobic amino acid), that direct proteins to coated pits (reviewed in [S19]). The presence of either motif in the cytoplasmic tail of a target protein is sufficient to direct internalization. The YppØ and dileucine motifs are directly recognized by different subunits of the clathrin adaptor complexes (APs) [S20–S23]. These large heterotetrameric complexes are responsible for recruiting sorted proteins into coated pits, and link cargo recruitment to clathrin coat assembly [S19]. AP-1 is primarily responsible for clathrin-mediated traffic from the trans-Golgi network (TGN), while AP-2 is responsible for traffic originating from the plasma membrane. AP-1 and AP-2 each contain two large chains (γ and β1 for AP-1, α and β2 for AP-2), a medium chain (μ1 or μ2) and a small chain (σ1 or σ2). The μ1/μ2 chain is the binding site for the YppØ motif [S20–S22], while the β1/β2 chain is responsible for binding the dileucine motif ([S23] and our unpublished observations).

Using a pull-down assay, it has recently been shown that Nef can interact with AP-1, raising the possibility that in cells Nef can interact directly with clathrin adaptors [S24]. Mutations in short sections in the two disordered loops of HIV-1 Nef have been shown to prevent localization to coated pits and CD4 downregulation but the sequences do not correspond to canonical sorting signals [S14]. It has been claimed that Nef interacts with APs through a tyrosine-based motif of the type YppØ in the amino-terminal arm, but the two motifs identified in that study (performed on SIV mac239 Nef) are absent in all HIV-1 strains (though similar tyrosine-based sequences are present in HIV-2 Nef) [S25]. This motif is therefore an unlikely candidate for the essential targeting motif directing Nef traffic, although it may have an auxiliary role in HIV-2 and SIV Nefs.

We therefore wished to determine whether other sorting signals are also present in Nef that might further explain Nef's ability to interact with clathrin adaptors and to traffic via the clathrin-mediated pathway. We found that a dileucine-containing peptide from the so-called carboxy-terminal disordered loop of Nef binds to the β chains of AP-1 and AP-2, as expected for an active dileucine-based internalization signal. This dileucine motif is highly conserved in the Nef sequence of HIV-1 strains. Mutation of this motif prevents Nef localization to clathrin-coated pits,

and also prevents the downregulation of CD4, but not of MHC class I molecules.

During the course of this study, it was reported that Nef also interacts with the 56 kDa subunit of the vacuolar membrane ATPase complex (V-ATPase) on the basis of experiments using the yeast two-hybrid system [S26]. The V-ATPase is required for acidification of endosomal compartments and also traffics via the clathrin-dependent pathway [S27,S28]. The region required for interaction between Nef and the 56 kDa subunit was mapped to $E_{178}D_{179}$ in HIV-2 SF2 (equivalent to $D_{174}D_{175}$ in HIV-1 NL4-3) at the carboxy-terminal boundary between the disordered loop and the core domain. A mutant HIV-1 NA7 Nef protein in which the amino acids equivalent to $D_{174}D_{175}$ were changed to alanines no longer localized to clathrin-coated pits [S14] and no longer caused downregulation of CD4 [S14,S26]. Antisense experiments have also been used to show that a decrease in the amount of the 56 kDa subunit reduces the rate of internalization of a chimeric protein composed of the ectodomain and transmembrane portion of CD8 fused to the Nef sequence [S26]. These results are consistent with the idea that the interaction between Nef and the V-ATPase is also required for the recruitment of Nef to clathrin-coated pits, and hence for CD4 downregulation. The downregulation of MHC class I molecules is not affected by this mutation, however [S29].

How does Nef induce the downregulation of MHC class I? At least three regions of Nef appear to be important in this process, an acidic motif in the amino-terminal arm, an Src homology 3 (SH3) ligand domain, and a portion of the carboxyl terminus [S24,S29]. Downregulation requires a YppØ motif in the cytoplasmic tail of the MHC class I heavy chain: in HLA-A and HLA-B, this motif has the sequence YSQA, [S24,S29] which would be expected to give weak interaction with the μ1-adaptin chain of AP-1. It is unclear whether this motif is involved in the normal traffic of MHC molecules. One possibility is that the YSQA motif is normally non-functional, and that Nef somehow induces a conformational change in the MHC class I cytoplasmic tail that allows this motif to be used for traffic [S24,S29]. A kinase or phosphatase, interacting with Nef via the SH3 ligand domain, might conceivably mediate this activation. Alternatively, Nef may cause the MHC molecules to aggregate, increasing the avidity of binding of clathrin-associated AP complexes to the YSQA motif.

The model that emerges from these studies is a complex one: Nef must contain at least four functional regions required for its effects on membrane traffic, one responsible for interacting with V-ATPase, the second containing an AP-binding motif, the third providing a direct or indirect interaction with CD4 and the fourth being required for MHC class I downregulation. From our results and those of Lu *et al.* [S26] it would appear that Nef requires both the V-ATPase interaction domain and the AP-binding motif to localize to coated pits and induce CD4 downregulation. These interactions may therefore offer an attractive novel target for drug design.

References

S1. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, *et al.*: **Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.** *Science* 1995, **270:**988-991.

S2. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC: **Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection.** *New Engl J Med* 1995, **332:**228-232.

S3. Kestler HWd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC: **Importance of the nef gene for maintenance of high virus loads and for development of AIDS.** *Cell* 1991, **65:**651-662.

S4. Schwartz O, Dautry-Varsat A, Goud B, Marechal V, Subtil A, Heard JM, Danos O: **Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes.** *J Virol* 1995, **69:**528-533.

S5. Garcia JV, Miller AD: **Serine phosphorylation-independent downregulation of cell-surface CD4 by nef.** *Nature* 1991, **350:**508-511.

S6. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D: **HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.** *Nature* 1998, **391:**397-401.

S7. Mariani R, Skowronski J: **CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals.** *Proc Natl Acad Sci USA* 1993, **90:**5549-5553.

S8. Mariani R, Kirchhoff F, Greenough TC, Sullivan JL, Desrosiers RC, Skowronski J: **High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection.** *J Virol* 1996, **70:**7752-7764.

S9. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM: **Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein.** *Nat Med* 1996, **2:**338-342.

S10. Anderson S, Shugars DC, Swanstrom R, Garcia JV: **Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells.** *J Virol* 1993, **67:**4923-4931.

S11. Pelchen-Matthews A, Armes J, Marsh M: **Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells.** *EMBO J* 1989, **8:**3641-3649.

S12. Chen BK, Gandhi RT, Baltimore D: **CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef.** *J Virol* 1996, **70:**6044-6053.

S13. Aiken C, Konner J, Landau NR, Lenburg ME, Trono D: **Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain.** *Cell* 1994, **76:**853-864.

S14. Greenberg ME, Bronson S, Lock M, Neumann M, Pavlakis GN, Skowronski J: **Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation.** *EMBO J* 1997, **16:**6964-6976.

S15. Grzesiek S, Bax A, Clore GM, Gronenborn AM, Hu J-S, Kaufman J, Palmer I, *et al.*: **The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase.** *Nat Struct Biol* 1996, **3:**340-345.

S16. Lee CH, Saksela K, Mirza UA, Chait BT, Kuriyan J: **Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain.** *Cell* 1996, **85:**931-942.

S17. Grzesiek S, Stahl SJ, Wingfield PT, Bax A: **The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR.** *Biochemistry* 1996, **35:**10256-10261.

S18. Mangasarian A, Foti M, Aiken C, Chin D, Carpentier JL, Trono D: **The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane.** *Immunity* 1997, **6:**67-77.

S19. Kirchhausen T, Bonifacino JS, Riezman H: **Linking cargo to vesicle formation - receptor tail interactions with coat proteins.** *Curr Opin Cell Biol* 1997, **9:**488-495.

S20. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, *et al.*: **Interaction of tyrosine-based sorting signals with clathrin-associated proteins.** *Science* 1995, **269:**1872-1875.

S21. Boll W, Ohno H, Songyang Z, Rapoport I, Cantley LC, Bonifacino JS, Kirchhausen T: **Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes.** *EMBO J* 1996, **15:**5789-5795.

S22. Rapoport I, Miyazaki M, Boll W, Duckworth B, Cantley LC, Shoelson S, Kirchhausen T: **Regulatory interactions in the recognition of endocytic sorting signals by AP-2 complexes.** *EMBO J* 1997, **16:**2240-2250.

S23. Rapoport I, Chen YC, Cupers P, Shoelson SE, Kirchhausen T: **Dileucine-based sorting signals bind to the beta chain of AP-1 at a site distinct and regulated differently from the tyrosine-based motif- binding site.** *EMBO J* 1998, **17:**2148-2155.

S24. Le Gall S, Erdtmann L, Benichou S, Berlioz-Torrent C, Liu L, Benarous R, *et al.*: **Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules.** *Immunity* 1998, **8:**483-495.

S25. Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, Trono D: **Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes.** *EMBO J* 1998, **17:**2472-2481.

S26. Lu X, Yu H, Liu SH, Brodsky FM, Peterlin BM: **Interactions between HIV-1 Nef and vacuolar ATPase facilitate the internalization of CD4.** *Immunity* 1998, **8:**647-656.

S27. Forgac M, Cantley L, Wiedenmann B, Altstiel L, Branton D: **Clathrin-coated vesicles contain an ATP-dependent proton pump.** *Proc Natl Acad Sci USA* 1983, **80:**1300-1303.

S28. Stevens TH, Forgac M: **Structure, function and regulation of the vacuolar (H+)-ATPase.** *Annu Rev Cell Dev Biol* 1997, **13:**779-808.

S29. Greenberg ME, Iafrate AJ, Skowronski J: **The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes.** *EMBO J* 1998, **17:**2777-2789.

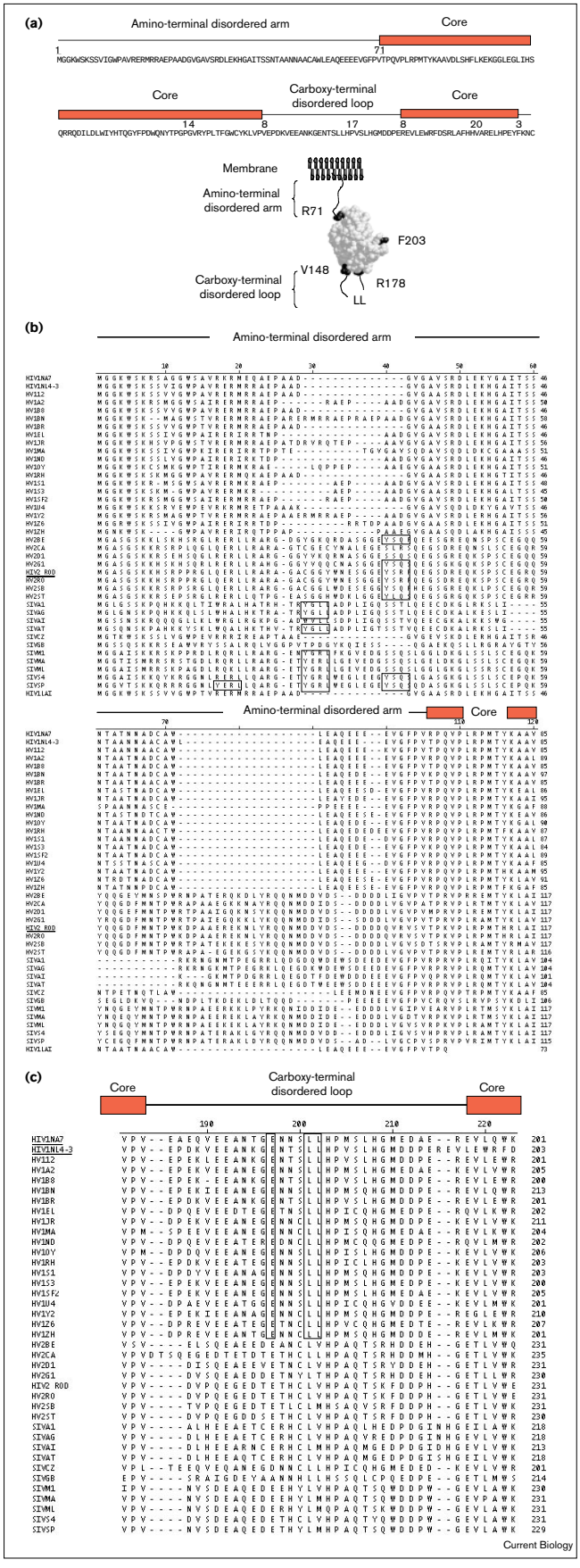

Figure 1

Conserved dileucine motif in Nef proteins. **(a)** Alignment of the amino acid sequence of HIV-1 NL4-3 Nef with the structural elements defined by its high-resolution structure [S15,S16]. The structural elements corresponding to the core, the amino-terminal disordered arm and the carboxy-terminal disordered loop are indicated. The amino acids located at the boundary between the core and the disordered segments are shown. R71 replaces T71 in the HIV-1 NL4-3 Nef protein used for its X-ray structural determination [S16]. **(b)** Alignment of the amino acid sequences from the amino-terminal disordered arm of representative Nef proteins of HIV-1, HIV-2 and SIV. Tyrosine-based motifs of the type YppØ are boxed. The HIV strain used for its functional identification is underlined. **(c)** Alignment of the amino acid sequences from the carboxy-terminal disordered loop of Nef proteins of HIV-1, HIV-2, and SIV. The conserved dileucine motif and the glutamic acid are boxed. The HIV strains NA7 and NL4-3 used in this paper are underlined.