Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis

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The simian immunodeficiency virus (SIV) and human immunodeficiency virus type 1 (HIV-1) Nef proteins induce the endocytosis of CD4 and class I MHC molecules. Here we show that SIV Nef interacts with the AP-2 adaptor complex via two elements located in the N-terminal region of the Nef molecule, but only the N-distal element is required to induce CD4 endocytosis. This N-distal AP-2 targeting element contains no canonical endocytic signals and probably contacts the AP-2 complex via a novel interaction surface. The data support a model where SIV Nef induces CD4 endocytosis by promoting the normal interactions between the di-leucine sorting signal in the CD4 cytoplasmic domain and AP-2, but does not substitute for the CD4–AP-2 adaptor interaction. Neither element is important for the induction of class I MHC endocytosis, thus indicating that different mechanisms underlie the induction of class I MHC and CD4 endocytosis by Nef. In contrast to SIV Nef, HIV-1 Nef interacts with AP-2 via a surface containing a di-leucine endocytosis signal in the C-terminal disordered loop of Nef. The fact that genetic selection maintains similar molecular interactions via different surfaces in SIV and HIV-1 Nef proteins indicates that these interactions have critical roles for the viral life cycle in vivo.

Keywords: CD4/clathrin/endocytosis/HIV/Nef

Introduction

Nef is a regulatory protein of human and simian immunodeficiency viruses (HIV and SIV) required for maximal viral virulence (for reviews see Emmerman and Malim, 1998; Frankel and Young, 1998). Natural HIV-1 and SIV Nef proteins have several conserved effects on signal transduction and protein sorting machineries that probably enhance viral virulence in vivo. One effect of Nef is to downregulate expression of the CD4 molecule from the cell surface (Garcia and Miller, 1991). CD4 is an essential component of the cellular receptor for HIV and SIV virions and a component of the antigen receptor on class II major histocompatibility complex (MHC)-restricted T lymphocytes (for review see Littman, 1996). Nef also downregulates expression of class I MHC complexes from the cell surface (Schwartz et al., 1996). Through this effect, Nef can protect infected cells from cytotoxic T lymphocytes in vitro and, probably, can help infected cells evade the immune response in the infected host (Collins et al., 1998).

Nef probably induces the endocytosis of CD4 via a clathrin-dependent pathway (Greenberg et al., 1997; Craig et al., 1998; Piguem et al., 1998). This possibility is supported by a striking pattern of co-localization of HIV-1 Nef with clathrin and with the AP-2 adaptor complex (Greenberg et al., 1997). The AP-2 adaptor complex recognizes and links sorting signals in cytoplasmic domains of transmembrane proteins to the clathrin coat, thereby recruiting these proteins to the endocytic machinery (for reviews see Kirchhausen et al., 1997; Marks et al., 1997; Schmid, 1997). HIV-1 Nef appears to bind AP-2 directly via a sequence in the Nef C-terminal disordered domain that contains a di-leucine motif (Bresnahan et al., 1998; Greenberg et al., 1998a), a sorting signal that is recognized by the β-subunits of AP-1 and AP-2 adaptor protein complexes (Rapoport et al., 1998). The di-leucine sequence in HIV-1 Nef is also required for the positive effect of Nef on the infectivity of HIV-1 particles (Miller et al., 1994; Spina et al., 1994), thus suggesting that the ability of Nef to enhance viral infectivity probably involves the ability of Nef to interact with the protein sorting machinery (Craig et al., 1998). The C-terminal disordered domain may also contain NBP1, a 56 kDa subunit of vacular ATPase, via a surface overlapping with, or adjacent to, that interacting with AP-2 (Lu et al., 1998). Since CD4 is redistributed in Nef-expressing cells to sites at the plasma membrane where clathrin AP-2 adaptor complexes are concentrated (Greenberg et al., 1997; Oldridge and Marsh, 1998), Nef probably directly promotes the recruitment of CD4 to coated pits, thereby accelerating the internalization of CD4 molecules from the cell surface.

HIV-1 Nef also induces the endocytosis of class I MHC complexes. The internalized class I MHC complexes accumulate in the trans-Golgi in vesicles coated with the AP-1 adaptor complex and are most likely sorted to lysosomes and degraded (Schwartz et al., 1996; Greenberg et al., 1998b; Le Gall et al., 1998b). Unlike CD4, class I MHC does not contain a di-leucine sorting signal, and downregulation of class I MHC by Nef does not involve the di-leucine motif in Nef, thus suggesting that HIV-1 Nef uses different mechanisms to promote class I MHC endocytosis and CD4 endocytosis (Greenberg et al., 1998a,b). Notably, the requirement for a tyrosine residue in the cytoplasmic domain of class I MHC heavy chains (Le Gall et al., 1998) and for the SH3 domain-binding surface in HIV-1 Nef suggests that Nef may use elements of the signal transduction machinery to modulate class I MHC endocytosis (Greenberg et al., 1998b).
Like HIV-1 Nef, SIV Nef also induces the endocytosis of CD4 and class I MHC (Benson et al., 1993; Le Gall et al., 1998). Since these effects are quantitatively similar between HIV-1 and SIV Nef, and because SIV Nef can substitute for HIV-1 Nef in additional assays (Iafrate et al., 1997; Sinclair et al., 1997; Alexander et al., 1998), HIV-1 and SIV Nef proteins are close functional homologs. The amino acid sequence of the structured core domain of HIV-1 Nef is well conserved in SIV Nef, and the two proteins probably share similar domain organization (Myers et al., 1993; Grzesiek et al., 1996; Lee et al., 1996). However, there is poor conservation of sequences between HIV-1 and SIV Nef outside of the structured core domain, namely in the regions corresponding to disordered domains of HIV-1 Nef, suggesting that the constraints that maintain these sequences in the two proteins may be different. This is supported by the observation that HIV-1 Nef contacts components of the AP-2 complex via sequences in its C-terminus (Bresnahan et al., 1997; Sinclair et al., 1997; Alexander et al., 1998). As shown in Figure 1, this chimeric 239·GFP protein resulted in dose-dependent downregulation of class I MHC to the endocytic pathway via the AP-2 complex. We also found that the deletion of di-leucine motifs in the C-terminus of 239-Nef (mutant 239(1–86)ΔN86·GFP). Subsequently, we found that the deletion of alanine A23 to asparagine N86 disrupted co-localization with AP-2 to residues 1–86 of 239-Nef [mutant 239(1–86)ΔN86·GFP]. These observations indicated that the signals contained in the N-terminal region of SIV Nef are necessary and sufficient to direct 239-Nef to the clathrin–AP-2 complex in vivo.

**Fig. 1.** SIV Nef-GFP fusion protein downregulates CD4 expression on the cell surface. Flow cytometric analysis of the effect of the 239-GFP fusion protein on surface CD4 in Jurkat T cells. Cells transiently expressing 239-GFP or GFP alone were cultured overnight, and expression of CD4 and GFP was determined simultaneously by two-color flow cytometry.

**Results**

**239-Nef co-localizes with the AP-2 adaptor complex**

To study the cellular distribution of the 239-Nef protein, we constructed a chimeric protein comprised of 239-Nef joined at the C-terminal end to a strongly fluorescing variant of the GFP reporter protein (Staubner et al., 1998). As shown in Figure 1, this chimeric 239-GFP protein retained the ability to downregulate CD4 expression on the cell surface, since transient expression of the 239-GFP fusion protein resulted in dose-dependent downregulation of CD4 expression on the surface of positively transfected (GFP-positive) cells. Thus, the 239-GFP fusion protein retains the functions of the native 239-Nef protein which are required to downregulate CD4 expression.

To assess whether 239-Nef co-localizes with adaptor complexes, the 239-GFP fusion protein was transiently expressed in IMR90 and in REF52 fibroblasts, and the location of the 239-GFP protein in the cell was revealed by direct GFP fluorescence. The location of the adapter protein complexes AP-1 and AP-2 was revealed by indirect immunofluorescence with the AP-1-specific anti-γ-adaptin monoclonal antibody (mAb) 100/3 (Ahle et al., 1988), an AP-2-specific anti-α-adaptin rabbit serum or with the anti-β1/β2 adaptin mAb 100/1, which is specific for both AP-1 and AP-2 (Ahle et al., 1988). As shown in panel 4 of Figure 2A–C, expression of the 239-GFP protein produced a punctate fluorescence pattern throughout the cell periphery, similar to that observed previously for the HIV-1 Nef–GFP fusion proteins (Greenberg et al., 1997, 1998a). The pattern of 239-GFP fluorescence showed a high degree of co-localization with the distribution of β- and α-adaptin components of AP-2 (Figure 2A–C, compare panels 4 and 5, and see panel 6 in Figure 2A and B). A smaller fraction of the 239-GFP protein overlapped the distribution of the Golgi AP-1 adaptor protein complex revealed with the AP-1-specific anti-γ-adaptin 100/3 mAb (Figure 2A–C, compare panels 4 and 5, and see panel 6 in Figure 2C); however, the resolution of the images does not allow us to establish with certainty whether this overlap reflects specific co-localization of 239-GFP with AP-1. We conclude that most of the SIV 239-Nef protein co-localizes with the plasma membrane AP-2 adaptor complex in the cell.

**Two elements in the N-terminal region of 239-Nef mediate co-localization with the AP-2 adaptor complex**

To determine which sequences direct 239-Nef to the AP-2 adaptor complex, we analyzed the cellular distribution of a set of 239-GFP fusion proteins bearing deletions and/or amino acid substitutions in Nef. The data from these studies are summarized in Figure 3 and selected results are shown in Figure 4. In preliminary experiments, we mapped the region of Nef required for the co-localization with AP-2 to residues 1–86 of 239-Nef [mutant 239(1–86)-GFP]. Subsequently, we found that the deletion of alanine A23 to asparagine N86 disrupted co-localization of 239-GFP with AP-2 [mutant 239(A23-S86)-GFP]. These observations indicated that the signals contained in the N-terminal region of SIV Nef are necessary and sufficient to direct 239-Nef to the clathrin–AP-2 complex in vivo.
also results in a wild-type fluorescence pattern (Figure 4, panel 1). First, we tested the effect of alanine substitutions for tyrosines Y28 and Y39, because these tyrosines previously had been implicated as an important functional sorting motif in 239-Nef that mediated binding to the μ-subunit of the AP-2 complex (Piguet et al., 1998). As shown in panel 2 of Figure 4, these mutations compromised the ability of 239(1–45)-GFP to co-localize with AP-2 [239(1–45,Y28A,Y39A)-GFP]. Next we tested the effect of deleting leucine L31 to aspartic acid D37. This deletion removes a potential di-leucine-based sorting signal at leucines L31 and L32; it also disrupts the putative tyrosine Y28-based signal by deleting L31, which occupies the position frequently occupied by a bulky hydrophobic residue in the YxxΦ class of tyrosine-based sorting signals (Owen and Evans, 1998; for a review see Kirchhausen et al., 1997). This mutation had a more severe effect than the tyrosine substitution and resulted in a diffuse distribution of the fusion protein in the cell [239(1–45,Δ31-37)-GFP; Figure 4, panel 3]. Surprisingly, however, when Y28A,Y39A or Δ31-37 were placed in the context of an N-terminal segment extending from M1 to N86 (Figure 4, panel 4), or in that of the full-length 239-Nef (panels 5 and 6), neither of these mutations disrupted the punctate distribution that co-localized with AP-2. Thus, in addition to the AP-2 targeting signal disrupted by mutations Y28A and Δ31-37, other sequences located in the S40–N86 N-terminal region of 239-Nef can target the protein to the AP-2 adaptor complex.

To map the other N-distal AP-2-targeting signal(s), additional mutations were combined with the Δ31-37 deletion in the background of 239(1-86,Δ31-37)-GFP, and in the background of the full-length 239-GFP fusion protein 239(Δ31-37)-GFP. Interestingly, deletions of amino acids Y65–P73 (see Figure 3) and Q64–N67 [Figure 4, panel 8, 239(Δ31-37,Δ64-67)-GFP] disrupted the punctate distribution of fusion proteins in the cell, when combined with the Δ31-37 deletion. It is of note that the Δ64-67 deletion alone, in the absence of the Δ31-37 deletion, had no effect on the punctate distribution of the fusion protein [Figure 4, panel 7; 239(Δ64-67)-GFP]. Several other deletions in the N-terminal region such as Δ54-57 or Δ88-102 had no detectable effect on the cellular distribution of 239-GFP fusion protein, when combined with the Δ31-37 deletion [239(Δ31-37,Δ54-57)-GFP, 239(Δ31-37,Δ88-96)-GFP, 239(Δ31-37,Δ98-102)-GFP]. Therefore, a second signal that directs co-localization with AP-2 is located between amino acid residues G57 and D88 in the S40–N86 N-terminal region of 239-Nef.

To determine more precisely the sequences in the N-terminal segment of Nef that direct co-localization of SIV Nef with AP-2, we expressed a set of 239-GFP molecules progressively deleted through this segment, and analyzed their cellular localization. We found that the first 38 amino acid residues of the N-terminal region of Nef are sufficient to direct co-localization with AP-2 [mutant 239(1–38)-GFP]. More extensive C-terminal deletions [mutants 239(1–33)-GFP and 239(1–27)-GFP] resulted in a loss of the punctate expression pattern and a diffuse distribution in the cell, suggesting that an AP-2 targeting signal requires residues between alanine A23 and glycine G38. Next, to map the putative targeting signal, mutations were introduced into this region in the context of a truncated 239(1–45)-GFP fusion protein (M1–G45), which, like the mutant 239(1–38)-GFP mentioned above, also results in a wild-type fluorescence pattern (Figure 4, panel 1). First, we tested the effect of alanine substitutions for tyrosines Y28 and Y39, because these tyrosines previously had been implicated as an important functional sorting motif in 239-Nef that mediated binding to the μ-subunit of the AP-2 complex (Piguet et al., 1998). As shown in panel 2 of Figure 4, these mutations compromised the ability of 239(1–45)-GFP to co-localize with AP-2 [239(1–45,Y28A,Y39A)-GFP]. Next we tested the effect of deleting leucine L31 to aspartic acid D37. This deletion removes a potential di-leucine-based sorting signal at leucines L31 and L32; it also disrupts the putative tyrosine Y28-based signal by deleting L31, which occupies the position frequently occupied by a bulky hydrophobic residue in the YxxΦ class of tyrosine-based sorting signals (Owen and Evans, 1998; for a review see Kirchhausen et al., 1997). This mutation had a more severe effect than the tyrosine substitution and resulted in a diffuse distribution of the fusion protein in the cell [239(1–45,Δ31-37)-GFP; Figure 4, panel 3]. Surprisingly, however, when Y28A,Y39A or Δ31-37 were placed in the context of an N-terminal segment extending from M1 to N86 (Figure 4, panel 4), or in that of the full-length 239-Nef (panels 5 and 6), neither of these mutations disrupted the punctate distribution that co-localized with AP-2. Thus, in addition to the AP-2 targeting signal disrupted by mutations Y28A and Δ31-37, other sequences located in the S40–N86 N-terminal region of 239-Nef can target the protein to the AP-2 adaptor complex.

To map the other N-distal AP-2-targeting signal(s), additional mutations were combined with the Δ31-37 deletion in the background of 239(1-86,Δ31-37)-GFP, and in the background of the full-length 239-GFP fusion protein 239(Δ31-37)-GFP. Interestingly, deletions of amino acids Y65–P73 (see Figure 3) and Q64–N67 [Figure 4, panel 8, 239(Δ31-37,Δ64-67)-GFP] disrupted the punctate distribution of fusion proteins in the cell, when combined with the Δ31-37 deletion. It is of note that the Δ64-67 deletion alone, in the absence of the Δ31-37 deletion, had no effect on the punctate distribution of the fusion protein [Figure 4, panel 7; 239(Δ64-67)-GFP]. Several other deletions in the N-terminal region such as Δ54-57 or Δ88-102 had no detectable effect on the cellular distribution of 239-GFP fusion protein, when combined with the Δ31-37 deletion [239(Δ31-37,Δ54-57)-GFP, 239(Δ31-37,Δ88-96)-GFP, 239(Δ31-37,Δ98-102)-GFP]. Therefore, a second signal that directs co-localization with AP-2 is located between amino acid residues G57 and D88 in 239-Nef and involves amino acids from glutamine Q64 to asparagine N67. Together, our observations indicate that SIV Nef is directed to membranes containing the AP-2 adaptor complex in the cell by at least two independent and apparently redundant signals located in the N-terminal region of the 239-Nef protein.

Both the N-proximal and the N-distal AP-2 targeting elements in the N-terminal region of 239-Nef mediate binding of AP-2 in vitro

It was reported previously that 239-Nef interacts with the μ2-adaptin subunit of the AP-2 complex in the yeast two-hybrid system and that this interaction is disrupted by mutating tyrosines Y28 and Y39 in the N-proximal AP-2 targeting element in Nef (Piguet et al., 1998). We also
detected an interaction between 239-Nef and full-length μ2-subunit using the two-hybrid system, but mutations that disrupted the N-proximal element did not disrupt the interaction with μ2 in a two-hybrid assay (data not shown). However, we found that these mutations disrupted the ability of the full-length 239-Nef protein to co-localize with AP-2 only when combined with a mutation of the N-distal element, suggesting that the N-distal element can also interact with one of the subunits of AP-2. Since we were not able to detect a specific interaction between individual subunits of AP-2 (such as α, β, μ or σ) and 239-Nef in the yeast two-hybrid system (data not shown), we asked whether 239-Nef can interact with native AP-2 complexes in vitro.

A chimeric protein comprising 239-Nef fused to GST (239-GST), or GST alone, were immobilized on glutathione–Sepharose and incubated with adaptor complexes isolated from clathrin-coated vesicles prepared from calf brains. The beads were washed extensively, the bound proteins were eluted from the glutathione–Sepharose and the AP-2 and AP-1 complexes were detected by immunoblotting with the AP-2-specific mAb 100/2 reacting with α-adaptin and with the AP-1-specific mAb 100/3 reacting with γ-adaptin (Ahle et al., 1988). The 239-GST fusion protein, but not GST alone, precipitated readily detectable amounts of the AP-2 and AP-1 adaptor complexes (Figure 5A and B, compare lane 6 with 5). The 239-GST protein containing only the N-terminal region of 239-Nef to the asparagine N86 bound AP-2 and AP-1 as efficiently as the full-length 239-Nef protein (compare lane 7 with 6). To verify further the relevance of the Nef–adaptor interaction detected in vitro, we analyzed the effect of mutation Y28A,Y29A and of deletions Δ31-37 and Δ64-67, which as shown above disrupted the co-localization of 239-Nef with AP-2 in vivo, on binding to AP-2 in vitro. Mutations that disrupted the N-proximal (Y28A,Y39A; Δ31-37) or N-distal (Δ64-67) AP-2 targeting element in Nef individually each decreased the binding of the AP-2 complex (Figure 5A, compare lanes 9, 10 and 11 with 6). Disruption of both elements by combining deletions Δ31-37 and Δ64-67 further decreased the binding of AP-2 (lane 8). This latter mutation completely disrupted the co-localization of 239-GFP with AP-2 in vivo. Thus there is a good correlation between the ability of 239-Nef to co-localize with AP-2 in vivo and to bind AP-2 in vitro, suggesting that Nef co-localizes with AP-2 by interacting
Fig. 4. Redundant elements in the N-terminal region of 239-Nef direct Nef to the AP-2 clathrin-adaptor complex. The effect of mutations in the N-terminal region of 239-Nef on the distribution of the 239-GFP fusion protein in the cell. The GFP fusion proteins were transiently expressed in fibroblasts and detected by direct GFP fluorescence (panels 1–8). β-adaptin was revealed by indirect immunofluorescence with mAb 100/1 (not shown).

directly with an AP-2-specific adaptin subunit, or with another factor that is tightly associated with the AP-2 complex. Notably, the mutations that disrupted the interaction of Nef with AP-2 had much less effect on the ability of Nef to bind AP-1; the combined Δ31-37/Δ64-67 deletions reduced binding to AP-1 by at most 25–35% (Figure 5B, compare lanes 6 and 8). This suggests that the interaction with the AP-1 complex in vitro may involve surfaces of the 239-Nef protein different from those that bind AP-2. A relatively low affinity interaction between 239-Nef and AP-1 in vitro is consistent with a relatively poor co-localization of 239·GFP and AP-1 in cells (see Figure 2C).

The 239-Nef–AP-2 interaction is dispensable for class I MHC downregulation

239-Nef induces the endocytosis of class I MHC complexes (Schwartz et al., 1996), and the interaction of 239-Nef with AP-2 could have a role in class I MHC downregulation. Therefore, we analyzed the role of the interactions between 239-Nef and the AP-2 adaptor complex in the induction of class I MHC complex endocytosis. To permit an accurate and sensitive comparison of the effect of the wild-type and mutant Nef proteins, we constructed a vector expressing Nef and the GFP protein, which here was used as a marker of transfected cells, from the same bi-cistronic transcription unit. This design results in a constant ratio of Nef and GFP reporter molecules expressed from a single bi-cistronic mRNA, thus allowing direct and reliable comparison of the activities of the native (unfused) wild-type and mutant 239-Nef proteins.

Transient transfection of the bi-cistronic vector expressing wild-type 239-Nef and GFP resulted in downregulation of class I MHC complexes that correlated with the increasing intensity of GFP fluorescence and thus increasing levels of Nef expression (Figure 6A, panel 2). Notably, neither of the mutations disrupting the interaction between 239-Nef and AP-2 in vivo and in vitro decreased the ability of Nef to downregulate expression of class I MHC complexes on the cell surface (Figure 6A, compare panels 3–5 with 2). Importantly, combining these mutations in the same 239-Nef molecule also did not have a detectable negative effect on class I MHC downregulation (panel 6).

To confirm these observations, we employed a fluorescence-activated cell sorting (FACS)-based internalization assay to compare the rates of class I MHC endocytosis in cells expressing similar levels of wild-type or mutant 239-Nef proteins (Figure 6B). In cells expressing wild-type 239-Nef, the rate of class I MHC internalization was increased ~3- to 5-fold compared with that in cells expressing the GFP reporter protein alone (compare GFP and 239). Nef proteins bearing mutations in the N-proximal or N-distal AP-2-binding element individually were at least as efficient as wild-type 239-Nef at promoting class I MHC endocytosis. These data indicate that the interactions of 239-Nef with the AP-2 complex mediated
by the N-terminal region of the SIV Nef protein are not required for induction of class I MHC internalization and class I MHC downregulation by Nef.

**Only the N-distal AP-2-binding element in 239-Nef is important for CD4 downregulation**

To assess the role of interactions between 239-Nef and the plasma membrane AP-2 adaptor in the downregulation of CD4 molecules from the cell surface, we tested the effect of selected mutations in the N-terminal region of Nef on its ability to alter steady-state CD4 expression on the cell surface. Since we observed previously that tyrosines Y28 and Y39 are important for the N-proximal element to co-localize with AP-2 in vivo, and since it had been reported that these tyrosines are critical for the ability of 239-Nef to induce endocytosis of CD4 (Piguet et al., 1998), we tested the effect of mutations in these residues on the ability of Nef to downregulate cell surface CD4 expression using native unfused 239-Nef proteins. Surprisingly, we found that alanine substitutions for tyrosines Y28 and Y39 had little, if any, detectable effect on the ability of 239-Nef to downregulate CD4 expression (Figure 7A, compare panel 5 with 2) or to accelerate CD4 internalization (Figure 7C). Similarly, the deletion Δ31-37, which removes the putative tyrosine-based endocytosis signal together with leucines L31 and L32 and adjacent sequences of the N-proximal element, also had no significant effect on the downregulation of steady-state CD4 expression (Figure 7C, panel 3) and notably also for CD4 internalization. To address the possibility that the N-proximal and N-distal elements have cell type-specific roles in the downregulation of CD4, possibly as a result of the expression of Lck protein tyrosine kinase in Jurkat T cells, similar experiments were performed using HeLa cells constitutively expressing the human CD4 molecule (Kimpton and Emerman, 1992). Again, as shown in Figure 7B, the mutations in the N-proximal AP-2 targeting element had little detectable effect on the ability of 239-Nef to downregulate CD4 surface expression, while mutating the N-distal element drastically reduced the ability of 239-Nef to downregulate CD4 expression. All mutant 239-Nef proteins were stable proteins because all retained wild-type activity in downregulating class I MHC expression and because their steady-state expression levels were similar to that observed with wild-type 239-Nef (Figure 7D). Therefore, we conclude that the N-proximal AP-2-targeting element in 239-Nef is not important for the ability of Nef to downregulate CD4 or to induce CD4 internalization.

In contrast, the disruption of the N-distal AP-2-interacting element in 239-Nef, by deleting residues 64–67, disrupted the ability of Nef to downregulate CD4 surface expression (Figure 7D, panel 4). Moreover, the disruption of the N-distal AP-2-interacting element compromised the ability of 239-Nef to accelerate the internalization of CD4 (Figure 7C). Therefore, we concluded that only the N-distal AP-2-targeting element in 239-Nef is important for the ability of Nef to induce CD4 internalization and to downregulate CD4.

**The N-distal AP-2 targeting element does not contain canonical tyrosine-based sorting signals**

Although the N-distal element does not contain a dileucine sequence, it does contain three conserved tyrosine residues, Y60, Y65 and Y82, thus raising the possibility that one or more tyrosine-based sorting signals in the N-distal element contact AP-2 and mediate CD4 endocytosis. In canonical tyrosine-based sorting motifs, the critical tyrosine residue is followed by a bulky hydrophobic amino acid residue at position Y+3 (Kirchhausen et al., 1997; Marks et al., 1997). Both residues usually are located within disordered regions and both residues make contacts with the acceptor site in the μ-adaptin subunit of the adaptor complex (Kirchhausen et al., 1997; Owen and Evans, 1998). None of the tyrosine residues Y60, Y65 or Y82 are followed by a bulky hydrophobic residue at the Y+3 position, and therefore they are not likely to form such sorting signals. Nevertheless, to assess the possible role of Y60, Y65 and Y82 as endocytosis signals, each tyrosine was mutated to alanine and the effect of the...
mutations on the downregulation of CD4 was measured using bi-cistronic GFP reporter plasmids.

As shown in Figure 8, each mutation disrupted the ability of 239-Nef to decrease steady-state CD4 surface expression (panels 8–10), indicating the importance of tyrosines Y60, Y65 and Y82 for CD4 downregulation. However, none of these mutations had a detectable effect on class I MHC downregulation (panels 3–5), indicating that these mutations did not grossly perturb the general folding or the stability of the mutant 239-Nef proteins. However, since tyrosine to alanine substitutions can be drastic changes that disrupt local folding, we also tested the effect of phenylalanine substitutions at these positions; phenylalanine differs from tyrosine only by the absence of a hydroxyl group. In tyrosine-based sorting motifs, it is this hydroxyl group that binds to Asp176, Lys203 and Arg423 at the μ-adaptin acceptor site (Owen and Evans, 1998). Biochemical studies have shown that tyrosine–phenylalanine substitutions reduce the affinity of tyrosine-based motifs for their ligand by ~100-fold (Boll et al., 1996). Therefore, phenylalanine substitutions should abrogate any interaction of 239-Nef with μ-adaptin, having a minimal effect on the normal folding of 239-Nef. Notably, we found that Y60F and Y65F substitutions had no detectable effect on the ability of Nef to downregulate steady-state CD4 expression (Figure 8, panels 11 and 12). Thus, alanine substitutions for Y60 and Y65 probably disrupt CD4 downregulation by altering the local conformation of the N-distal AP-2-targeting element, rather than by inactivating a tyrosine-based endocytic signals in 239-Nef. The Y82F substitution only partially disrupted the ability of 239-Nef to downregulate CD4 expression (Figure 8, panel 13). This suggests that the hydroxyl group of tyrosine Y82 may have some role in CD4 downregulation, even though it is not likely to be part of a canonical tyrosine-based sorting motif.

The N-terminal region of 239-Nef is important for SIV infectivity

Nef stimulates the infectivity of HIV-1 and SIV particles, and this effect is probably responsible to a large extent for the enhanced replication of viruses containing an intact Nef gene in peripheral blood mononuclear cells (PBMC) (Miller et al., 1994; Spina et al., 1994). To assess the effect of mutations in the AP-2-targeting elements in 239-Nef on SIV replication, the critical mutant nef alleles were introduced into SIV and the infectivity of the resulting viruses was determined in a single round replication assay with a CD4-positive indicator cell line (sMagi; Chackerian et al., 1995; Lang et al., 1997). These cells contain an integrated lacZ reporter gene under transcriptional control of the SIV long terminal repeat (LTR). The productive infection of sMagi cells results in the activation of the lacZ reporter, thus permitting the detection and quantitation of infected cells. As shown in Figure 9, the infectivity of SIV particles containing an intact 239-Nef was ~8-fold higher than that of the virus containing a prematurely terminated Nef-coding sequence (compare 239 with 239Δ). Deletion of amino acids 23–74 in 239Δ-Nef disrupted the positive effect of 239-Nef almost completely. Mutations disrupting the N-proximal AP-2-targeting element by deleting amino acids 31–37 [see 239(Δ31-37)] in Figure 5], or by substituting tyrosines Y28 and Y39 with alanines [see 239(Y28A,Y39A)] or the N-distal element [see 239(Δ64-67)] individually did not have a negative effect, but rather enhanced the infectivity (Figure 9). In contrast, combining the mutations in both elements severely reduced the positive effect of 239-Nef on SIV infectivity by ~50% [see 239(Δ31-37,Δ64-67)]. These observations indicate that the N-terminal region of 239-Nef containing the N-proximal and N-distal AP-2-interacting elements is required for the positive effect of 239-Nef on SIV particle infectivity. They are also consistent with the
possibility that the regions containing the AP-2-interacting elements in 239-Nef have a role in the effect of 239-Nef on SIV infectivity, but the interaction of 239-Nef with AP-2 is not a rate-limiting event for the enhancement of SIV replication, and that an additional important interaction(s) is probably mediated by an adjacent surface in the N-terminal region of 239-Nef. Our observation is consistent with the observation from HIV-1 Nef, where an amino acid change that disrupts the interaction with the AP-2 complex (Greenberg et al., 1998) also reduces the positive effect of Nef on replication and infectivity (Craig et al., 1998) and thereby links these two functions to the same region of the 239-Nef molecule.

Discussion

We have observed that 239-Nef, like HIV-1 Nef, co-localizes with the AP-2 adaptor complex. Unlike co-localization of HIV-1 Nef, which requires an element in the Nef C-terminal loop, co-localization of 239-Nef with the AP-2 adaptor complex is mediated independently by two elements located within the N-terminal segment of the 239-Nef molecule. An N-proximal AP-2-targeting element is located between tyrosine Y29 and glycine G38, and an N-distal element involves sequences between glutamine Q58 and aspartic acid D88. Notably, mutations in the two elements also diminish the ability of 239-Nef to associate with AP-2 in vitro. Our evidence suggests that each element must form an autonomously folded surface that independently interacts with a component(s) of the AP-2 adaptor complex. That SIV Nef can interact with AP-2 through two extended surfaces of the SIV Nef molecule is a likely explanation for the remarkable co-localization of 239-Nef with the AP-2 adaptor complex in the cell.

Mutations in SIV 239-Nef that disrupt its interactions with the AP-2 adaptor complex in vivo and in vitro disrupt the ability of 239-Nef to induce the endocytosis of CD4, but not class I MHC. These data agree with the previous
Fig. 8. The effect of tyrosine mutations in the N-distal AP-2-targeting element in 239-Nef on the ability to downregulate CD4 and class I MHC surface expression. The effect of mutations in 239-Nef on the steady-state CD4 (lower panel) and class I MHC (upper panel) expression on the cell surface was determined as described in the legends to Figures 6 and 7.

observation (Greenberg et al., 1998a,b) that mutations in the C-terminal loop in HIV-1 Nef which abolish the interaction(s) of HIV-1 Nef with AP-2 disrupt the ability of Nef to downregulate CD4, but not class I MHC expression. The data also suggest that the downregulation of class I MHC and CD4 molecules involves different mechanisms.

Our data from experiments with native 239-Nef protein indicate that the N-distal, but not the N-proximal, AP-2 targeting element is critical for the downregulation of surface CD4 expression and the induction of CD4 endocytosis, and, therefore, suggest different functional roles for the N-proximal and N-distal AP-2-interacting elements in 239-Nef. It is of note that the critical role of the N-distal element in CD4 downregulation was not detected by the previous study of SIV Nef interaction with AP-2, which was based to a large extent on results from chimeric proteins containing 239-Nef fused to the transmembrane and extracellular portions of an integral membrane protein (Piguet et al., 1998). While such fusions have been invaluable in the analysis of sorting signals located in cytoplasmic domains of integral membrane proteins, it is possible that some aspects of the interactions between Nef and adaptors are not reproduced faithfully with such fusion proteins.

Why the interaction of 239-Nef with AP-2 via the N-proximal element is not sufficient to induce CD4 endocytosis in the absence of the N-distal element is not known. One possible explanation for these observations is that the interaction involving the N-distal element, but not the N-proximal element, re-orient the 239-Nef molecule with respect to the AP-2 complex in a very specific way that promotes the recruitment of CD4 to the AP-2 complex and the induction of CD4 endocytosis. Alternatively, the N-distal element in the N-terminal loop of SIV Nef may have an additional function besides linking Nef to the AP-2 complex, such as a direct or indirect interaction with CD4 or perhaps with other molecules that control the recruitment of CD4 and/or the morphogenesis of the coated pit. Finally, it is possible that the N-proximal element is important for other sorting proteins.
events that involve CD4, perhaps in the context of other adaptor complexes such as, for example, AP-3, or that it is involved in another function such as the sorting of membrane proteins other than CD4.

Unlike HIV-1 Nef, a di-leucine sequence is not required in 239-Nef for the interaction of 239-Nef with the AP-2 complex. In contrast, the di-leucine motif located in the CD4 cytoplasmic domain is required for the down-regulation of CD4 by 239-Nef (Hua and Cullen, 1997) and for CD4 internalization induced by phorbol esters (Pelchen-Matthews et al., 1993; Marsh and Pelchen-Matthews, 1996). Both these modes of CD4 internalization are probably mediated by an interaction between the di-leucine signal in CD4 and the acceptor site in the β-adaptin subunit of the AP-2 complex (Rapoport et al., 1998).

Since the N-distal AP-2 targeting element in 239-Nef does not contain a di-leucine sorting signal, it probably contacts a different surface of the AP-2 complex. Therefore, 239-Nef induces CD4 endocytosis probably by promoting the normal recruitment mechanism of the CD4 molecule to the endocytic machinery, rather than by substituting for the normal interaction between CD4 and AP-2. It is possible that 239-Nef modifies the AP-2 complex to increase its affinity for CD4 and/or by providing additional specific contacts with the CD4 cytoplasmic domain.

It is evident that the interactions with AP-2 map to different domains in the SIV and HIV-1 Nef proteins. In SIV Nef, the main determinant of this interaction maps to the N-terminal region of the protein. In contrast, with HIV-1 Nef, this determinant maps to the C-terminal loop because (i) a peptide derived from the C-terminal loop and containing a di-leucine sequence can be cross-linked to the β-subunits of the AP-2 complex (Greenberg et al., 1998a); (ii) in functional assays, this di-leucine sequence has the characteristics of a di-leucine-based sorting motif (Bresnahan et al., 1998; Craig et al., 1998); and (iii) the N-terminal loop of HIV-1 Nef is not sufficient to direct GFP to the AP-2 complex in the cell and the deletion of almost the entire N-terminal loop does not completely disrupt co-localization with AP-2 (Greenberg et al., 1997).

Interestingly, previous evidence suggested additional differences in the functional organization of HIV-1 and SIV Nef molecules in the core domains of the two molecules. For example, the PPII helix in HIV-1 Nef is essential for its ability to modulate CD3-initiated signaling in T cells and to induce endocytosis of class I MHC molecules, but not for the ability of SIV Nef to perform these functions (Lafra et al., 1997; Greenberg et al., 1998b). The observation that HIV-1 and SIV Nef proteins have evolved different surfaces to carry out similar functions is striking. Importantly, the observation of genetic selection to maintain similar molecular interactions via different surfaces in SIV and HIV-1 Nef proteins provides additional strong evidence that these interactions have critical roles in the viral life cycle in vivo.

Materials and methods

Plasmid construction

The oligo-directed site-specific mutagenesis of SIV mac239 nef was performed as described previously (Mariani and Skowronski, 1993; Lafra et al., 1997). All mutations were verified by DNA sequencing, and mutant alleles were subcloned into T cell-specific pCD3-β and/or CMV-based pCG expression vectors, as described previously (Tanaka and Herr, 1990; Skowronski et al., 1993; Greenberg et al., 1997). Genes directing expression of the 239-GFP fusion protein were constructed with the aid of PCR using standard subcloning techniques (Sambrook et al., 1989; Greenberg et al., 1997; Staufer et al., 1998). The bicistronic expression vectors containing 239-Nef followed by GFP under translational control of the EMCV internal ribosome entry site (IRES) element were constructed by subcloning the SIV 239-Nef protein-coding region followed by the EMCV IRES controlling the GFP-encoding sequence, immediately downstream of the CMV promoter in the pCG eukaryotic expression vector (Tanaka and Herr, 1990), using standard techniques (Sambrook et al., 1989) as described previously (Greenberg et al., 1998b). This arrangement should result in expression of various 239-Nef proteins in a constant stoichiometry with the GFP reporter molecules from a single bicistronic mRNA. Genes expressing mac239 Nef and GFP were kindly provided by R.C. Desrosiers and G.N. Pavlakis, respectively.

Cell lines and DNA transfections

Jurkat T cells expressing high levels of CD4 (JJK cells provided by D.R. Littman) were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES (pH 7.4) and 10% fetal bovine serum (FBS) and were culled 1:20–1:30 every 3 days (Lafra et al., 1997). REF52, IMR90 fibroblasts, Magi and SIV-Mag cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and subcultured 1:3 every 3–4 days. JJK, REF52 and Magi cells were transfected by electroporation as described previously (Lafra et al., 1997; Greenberg et al., 1998b). Briefly, cells were electroporated at 200 V and 960 μF with a total of 10–20 μg of DNA containing various amounts of appropriate expression vectors. Following transfection, the cells were cultured for an additional 16–24 h prior to flow cytometric or biochemical analyses of CD4, class I MHC, Nef, GFP and/or 239-GFP expression. For microscopic analysis, REF52 cells were plated on coverslips and cultured for an additional 24–48 h prior to the analysis.

Flow cytometry analysis

Flow cytometry analysis of CD4, or class I MHC molecules, and GFP reporter molecules in cells transfected with a bicistronic transcription unit expressing 239-Nef and GFP was performed on an Epics-Elite flow cytometer as described previously (Greenberg et al., 1997, 1998b). Briefly, aliquots of 2×10^5 cells were reacted with saturating amounts of phycoerythrin (PE)-conjugated mAb Leu3A, specific for human CD4 (Becton and Dickinson), or biotinylated B9.12.1 mAb, specific for the assembled class I MHC heavy chain β2-microglobulin complex (Immunotech), followed by PE-conjugated streptavidin (Caltag), in phosphate-buffered saline (PBS) containing 1% FBS and 0.1% sodium azide (PBS-FA). For analysis of CD4 and class I MHC expression, cells transfected with the bicistronic vector expressing 239-Nef and GFP were reacted with either PE-conjugated Leu3A mAb or biotinylated B9.12.1 mAb followed by PE-streptavidin conjugate, and expression of these molecules was detected by two-color flow cytometry on an Epics-Elite flow cytometer or on a FACSCalibur flow cytometer (Greenberg et al., 1998b). The level of CD4 or class I MHC expression (red fluorescence) was measured as a function of GFP fluorescence (green fluorescence).

CD4 and class I MHC endocytosis assays

Jurkat T cells, or HeLa cells expressing the human CD4 molecule (Magi; Kimpton and Emerman, 1992), were transfected with bicistronic plasmids expressing wild-type or mutant mac239 Nef proteins and GFP, and endocytosis of CD4 or class I MHC was characterized by flow cytometry as described previously (Greenberg et al., 1997). Briefly, 24 h after transfection, 5×10^5 cells were reacted with PE-conjugated Leu3A mAb or PE-conjugated mAb G46-2.6 specific for class I MHC heavy chain (PharMingen) for 0 min on ice in RPMI 1640 medium, or DMEM medium for Magi cells, containing 0.2% BSA, 10 mM HEPES (pH 7.4). Following removal of excess unbound antibody, aliquots of 10^6 cells were incubated for the indicated times at 37°C. The reactions were terminated on ice and each sample was then divided into two aliquots and diluted 5-fold with PBS, or with RPMI 1640 adjusted to pH 2 (acid wash to remove mAb that had not been internalized). Total CD4 (class I MHC) and internalized CD4 (class I MHC) were determined by flow cytometry for cells showing identical levels of GFP expression. The fraction of internalized CD4 (class I MHC) was determined as described previously (Greenberg et al., 1997).
**Fluorescent microscopy analysis**

REF52 cells, grown on cover slips, were fixed in 3% formaldehyde in PBS for 20 min at room temperature and permeabilized in 0.1% NP-40 in PBS. Following incubation for 30 min in blocking solution (3% BSA, 0.1% NP-40 in PBS), cover slips were incubated for 1 h with 100/1 mAb, specific for β-adaptins (Ahle et al., 1988; obtained from Sigma). Cover slips were then washed in 0.1% NP-40 in PBS, followed by a 30 min incubation with Texas Red-conjugated goat anti-mouse IgG antibody (Amersham), and washed in PBS before mounting on glass slides in glycerol-based mounting medium. Fluorescence microscopy images were taken with a Nikon Microphot-FXN microscope equipped with a CCD camera and processed using Oncor Imaging software as described previously (Greenberg et al., 1997). Alternatively, after blocking, cover slips were incubated with a rabbit serum specific for α-adaptin (kindly provided by Tom Kirchhausen; Greenberg et al., 1998a) followed by Texas Red-conjugated goat anti-rabbit IgG antibody.

**Recombinant proteins**

Genes expressing SIV mac239 Nef-GST fusion proteins were constructed using PCR and subcloned into the Escherichia coli expression vector pSBET (Shenk et al., 1995) using standard molecular biology techniques (Sambrook et al., 1989). The appropriate expression vectors were transformed into E.coli strain BL21 (DE3) (Studier et al., 1990) and expression of various 239-GST fusion proteins was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18°C. Cells were lysed and fusion proteins were purified on glutathione-Sepharose beads (Pharmacia) according to the manufacturer’s instructions. The yields of purified recombinant fusion proteins were estimated by comparison of serially diluted aliquots of the 239-GST fusion proteins with known amounts of BSA, separated on SDS-PAGE gels and visualized by staining with Coomassie Brilliant Blue.

**In vitro binding assays and immunoblot analysis of 239-Nef and adaptin subunits**

AP-1 and AP-2 adaptor protein complexes were partially purified from calf brains by several rounds of low speed and high speed centrifugation as described previously (Campbell et al., 1984; Matsui and Kirchhausen, 1990) and the extracts were quantitated using BCA reagent (Pierce). Aliquots (100 μl) of the appropriate 239-GST fusion protein were incubated with 200 μg aliquots of partially purified preparations of the adaptor protein complexes prepared from calf brains in a total volume of 200 μl of HEMGN buffer [12.5 mM HEPES pH 7.9, 7.5 mM KCl, 6.5 mM MgCl2, 0.05 mM EDTA, 2.5% glycerol, 0.05% NP-40, 1 mM dithiothreitol (DTT)] for 4 h at room temperature. Beads were washed five times for 5 min each with 2× concentrated HEMGN buffer and the bound proteins were eluted by boiling in reducing sample loading buffer. The amount of AP-1 and AP-2 adaptor protein complexes in the bead eluate was estimated by immunoblot analysis with the mAb 100/3, reacting with γ-adaptin subunit specific for the AP-1 adaptor protein complex (Ahle et al., 1988; Sigma), and with mAb 100/2, reacting with the α-subunit specific for the AP-2 adaptor protein complex (Ahle et al., 1988; Sigma). Small aliquots of the binding reactions were separated on SDS-polyacrylamide gels and analyzed by immunoblotting with mAbs specific for α- and γ-adaptin and for γ-adaptin to confirm that they contained equivalent amounts of partially purified adaptor protein complexes, or analyzed by staining with Coomassie Blue to confirm that they contained equivalent amounts of the wild-type and mutant 239-GST fusion proteins. Immunoblot analysis of 239-Nef expression in transfected cells was performed as described previously (Greenberg et al., 1997; Lang et al., 1997).

**SIV infectivity assays**

Mutant and wild-type nef alleles were subcloned immediately downstream of the env gene in the pBRANU plasmid containing the SIV mac239 provirus with a 513 bp deletion in the nef LTR region and mutations in the SIV nef initiation codon and a second in-frame ATG at codon 7 of the nef ORF (Gundlach et al., 1997; F.Kirchhoff, unpublished). Viral stocks were generated by 293T cells and p27 antigen concentration was determined as described previously (Lang et al., 1997). The infectivity of viral stocks in sMagi cells (Chackerian et al., 1995) was assayed as described previously (Lang et al., 1997).

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**References**


