Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation

Michael E.Greenberg, Scott Bronson,
Martin Lock, Markus Neumann¹,
George N.Pavlakis¹ and
Jacek Skowronski²

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and
¹ABL Basic Research Program, NCI-FCRDC, Frederick, MD 21702,
USA

²Corresponding author

The *nef* gene of human and simian immunodeficiency viruses is critical for AIDS pathogenesis. Its function *in vivo* is unknown, but *in vitro* natural isolates of Nef down-regulate expression of the cell surface CD4 molecule, a component of the T cell antigen receptor and the viral receptor, by accelerating its endocytosis. We have used chimeric proteins comprised of the natural HIV-1 NA7 Nef fused to a strongly fluorescing mutant of green fluorescent protein (GFP) to correlate Nef function with intracellular localization in human CD4-positive Jurkat T cells. The NA7-GFP fusion protein co-localizes with components of the clathrin coat, including clathrin and the β -subunit of the AP-2 adaptor protein complex, at discrete locations that are consistent with the normal cellular distribution of clathrin coats at the plasma membrane. The NA7-GFP protein is also found in the perinuclear region of the cell, which is likely to reflect the Golgi apparatus. Evidence from a CD4-negative fibroblast cell line indicates that co-localization of NA7-GFP with components of the clathrin coat does not require expression of the CD4 molecule. Analysis of a large panel of chimeric molecules containing mutant Nef moieties demonstrated that the N-terminal membrane targeting signal cooperates with additional element(s) in the disordered loops in the Nef molecule to co-localize the Nef protein with AP-2 adaptor complexes at the cell margin. This localization of NA7-GFP correlates with, but is not sufficient for, down-regulation of surface CD4 and at least one additional function of Nef is required. In T cells co-expressing CD4 and NA7-GFP, CD4 at the cell surface is redistributed into a discrete pattern that co-localizes with that of NA7-GFP. Our observations place NA7-GFP in physical proximity to AP-2-containing clathrin coat at the plasma membrane and imply that Nef interacts, either directly or indirectly, with a component of the AP-2-containing coat at this location. This evidence supports a model whereby Nef recruits CD4 to the endocytic machinery via AP-2-containing clathrin coats at the plasma membrane.

Keywords: adaptor complex/CD4/endocytosis/
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Introduction

The *nef* gene of human and simian immunodeficiency viruses (HIV and SIV) is an important determinant of viral pathogenicity in human and simian AIDS (Kestler *et al.*, 1991; Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995; Mariani *et al.*, 1996). Viral loads remain low and AIDS does not develop in rhesus macaques experimentally infected with a *nef*-deleted SIV variant (Kestler *et al.*, 1991). Moreover, a lack of disease progression in a subset of HIV-1-infected persons is associated with deletions in *nef* (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995).

The critical *in vivo* function(s) of Nef is not known. *In vitro* two major effects on cell function have consistently been observed with natural *nef* alleles. One effect is to induce alterations in cellular signal transduction pathways and the other is to down-regulate surface expression of the CD4 and MHC class I molecules. The ability of Nef to alter signal transduction has been shown by a Nef-mediated block of IL-2 gene and CD69 gene induction that follows stimulation via the T cell antigen receptor complex (TCR) in Jurkat T cells (Luria *et al.*, 1991; Baur *et al.*, 1994; Iafrate *et al.*, 1997). Nef has also been shown to alter platelet-derived growth factor receptor signaling in Nef-transfected NIH 3T3 fibroblasts and to have effects on cellular transformation in NIH 3T3 fibroblasts (De and Marsh, 1994; Du *et al.*, 1995; Graziani *et al.*, 1996). Some of these effects of Nef on signal transduction map to different amino acid residues and likely involve different interactions between Nef and components of the cell signal transduction machinery (Du *et al.*, 1995; Iafrate *et al.*, 1997). Candidate cellular protein partners for these interactions include an Src family protein tyrosine kinase or other SH3 domain protein and/or a serine kinase from the p21-activated kinase (PAK) family (Sawai *et al.*, 1994; Collette *et al.*, 1995; Saksela *et al.*, 1995; Lee *et al.*, 1996; Nunn and Marsh, 1996; Iafrate *et al.*, 1997).

The other well-established effect of Nef is down-regulation of cell-surface expression of CD4, an essential component of both the immunodeficiency virus receptor and of the TCR complex on MHC class II restricted T cells (Garcia and Miller, 1991). The ability of Nef to down-regulate CD4 surface expression is separable from its ability to block CD3 signaling in T cells (Iafrate *et al.*, 1997). The effect of Nef on CD4 surface expression could disrupt TCR recognition of MHC class II peptide complexes and a range of subsequent antigen-dependent signal transduction pathways. Indeed, in transgenic mice a 2- to 3-fold decrease in CD4 expression on the surface of immature double positive thymocytes expressing HIV-1 Nef is associated with compromised positive selection of CD4⁺ T lymphocytes (Skowronski *et al.*, 1993). Interference with T cell responses could benefit replication and/or survival of the virus in the infected host. Other possible

roles for Nef-induced down-regulation of surface CD4 include the protection of infected T cells from cytopathic effects of envelope-CD4 interactions (Schwartz *et al.*, 1993), promotion of viral particle release and prevention of superinfection (Benson *et al.*, 1993). Each of these effects may promote replication and/or survival of the virus *in vivo*.

Down-regulation of CD4 surface expression is a conserved function of Nef, since it has been observed with natural isolates of both SIV and HIV-1 Nef proteins (Anderson *et al.*, 1993; Benson *et al.*, 1993; Mariani and Skowronski, 1993). This effect of Nef involves accelerated internalization of the CD4 molecule from the cell surface, since Nef does not have a detectable effect on CD4 synthesis, on CD4 transport from the endoplasmic reticulum through the Golgi stacks or on sorting to the plasma membrane (Aiken *et al.*, 1994; Rhee and Marsh, 1994; Sanfridson *et al.*, 1994). Once internalized, CD4 molecules accumulate in early endosomes and are degraded in an acidic compartment (Aiken *et al.*, 1994; Rhee and Marsh, 1994; Sanfridson *et al.*, 1994; Schwartz *et al.*, 1995).

While the precise mechanism by which Nef induces CD4 internalization is not known, elements in CD4 that are required for its down-regulation are known. This effect of Nef requires four hydrophobic amino acid residues, including a di-leucine motif, in the membrane-proximal region of the CD4 cytoplasmic domain (Aiken *et al.*, 1994; Salghetti *et al.*, 1995). The di-leucine motif, similar to endocytosis and lysosomal sorting signals found in several cell surface proteins, is also required for CD4 endocytosis induced by phorbol esters, a process which involves CD4 recruitment to clathrin-coated pits (Pelchen-Matthews *et al.*, 1993; Marsh *et al.*, 1997). The residues in the cytoplasmic domain of CD4 that are required for the effect of Nef are also required for association of CD4 with the src family protein tyrosine kinase Lck (Anderson *et al.*, 1994; Salghetti *et al.*, 1995). Association of CD4 with Lck prevents association of CD4 with coated pits and blocks its endocytosis (Pelchen-Matthews *et al.*, 1993; Marsh *et al.*, 1996). The dissociation of CD4-Lck complexes precedes and is essential for CD4 internalization induced by phorbol esters (Sleckman *et al.*, 1992). Nef also disrupts association of CD4 with Lck, suggesting that Nef directly or via recruitment of cellular factors competes with Lck for a binding site in the CD4 cytoplasmic domain to induce CD4 endocytosis (Anderson *et al.*, 1994; Salghetti *et al.*, 1995).

Recruitment of transmembrane proteins to the clathrin coat involves recognition of sorting signals in the cytoplasmic domains of these proteins by components of the coat. These events are mediated by multisubunit adaptor protein complexes (AP) that interact directly with both clathrin and cytoplasmic domains of membrane receptors (Marks *et al.*, 1997; Robinson, 1997; Traub, 1997). Two types of adaptor complexes, AP-1 and AP-2, are known to mediate protein sorting from the plasma membrane (AP-2) and in the trans-Golgi network (AP-1 and AP-2) in association with clathrin. Subunits of the AP-1 and AP-2 complex are known to interact directly and specifically with one class of sorting signals that contain tyrosine-based motifs (Ohno *et al.*, 1995; Marks *et al.*, 1997). The components of the clathrin coat that interact with the

di-leucine-based endocytosis signals, such as that in CD4, are not known.

The ability of Nef to down-regulate CD4 was mapped to two disordered loops in the Nef molecule (Grzesiek *et al.*, 1996a; Lee *et al.*, 1996; Iafrate *et al.*, 1997) and to a surface in the structured core of the Nef protein (Hua *et al.*, 1997; Liu *et al.*, 1997). In this report we investigate the molecular interactions of Nef required for CD4 down-regulation. Using an HIV-1 Nef protein fused to green fluorescent protein (GFP; Prasher *et al.*, 1992; Chalfie *et al.*, 1994) to correlate the function and subcellular localization of Nef, we find that: (i) the Nef-GFP fusion protein is concentrated at structures that contain the β -subunit of the adaptor protein complex (β -adaptin) and clathrin at the cell margin and is also found in a perinuclear compartment; (ii) CD4 molecules expressed at the cell surface also co-localize with the structures containing Nef-GFP fusion protein at the cell margin; (iii) co-localization of Nef with β -adaptin does not require expression of the CD4 molecule; (iv) sequences within the two disordered loops of Nef are necessary to co-localize Nef with β -adaptin; (v) the ability of Nef to co-localize with β -adaptin at the cell margin correlates with ability to down-regulate cell surface expression of CD4; (vi) at least one additional interaction of Nef is required for CD4 down-regulation. Our results define novel molecular interactions between Nef and the endocytic machinery, indicate specific roles of various domains of the Nef protein in these interactions and suggest a mechanistic model for Nef-induced CD4 endocytosis.

Results

HIV-1 Nef-GFP fusion protein down-regulates CD4 expression at the cell surface

To investigate the subcellular localization of Nef we fused the C-terminal end of the NA7 Nef protein to the N-terminal end of GFPsg25, a strongly fluorescing mutant of GFP (Palm *et al.*, 1997). The NA7 Nef protein, encoded by a natural HIV-1 *nef* allele, was previously shown to strongly down-regulate the CD4 molecule from the cell surface (Mariani and Skowronski, 1993; Iafrate *et al.*, 1997). The ability of the NA7-GFP fusion protein to down-regulate surface CD4 was studied using a transient expression assay in the JJK CD4⁺ subline of Jurkat T cells. CD4 surface expression and GFP expression in the transfected JJK cells were quantitated simultaneously on a cell-by-cell basis using flow cytometric analysis.

As shown in Figure 1A, transient transfection of NA7 *nef* resulted in a 20- to 50-fold decrease in CD4 expression on the surface of positively transfected cells (compare panels Vector and NA7). Transfection of the GFP expression vector resulted in GFP fluorescence in a large fraction of cells and had no noticeable effect on CD4 expression (GFP panel). Transfection of the NA7-GFP expression vector resulted in both GFP fluorescence and a dramatic down-regulation of CD4 expression on the surface of GFP-positive cells (compare panel NA7-GFP with GFP and NA7). The decrease in surface CD4 was proportional to the amount of NA7-GFP fusion protein expressed in the transfected cells.

As shown in Figure 1B, the NA7-GFP fusion protein was active in promoting CD4 endocytosis, as reported

Fig. 1. HIV-1 Nef-GFP fusion protein down-regulates CD4 expression on the cell surface. (A) Flow cytometric analysis of the effect of NA7-GFP protein on surface CD4. Cells transfected with 20 µg plasmids expressing the HIV-1 NA7 Nef (NA7), GFP (GFP), NA7-GFP fusion protein or transfected with a control empty vector (Vector) were cultured overnight and CD4 expression on the cell surface and GFP expression were analyzed simultaneously by two color flow cytometry. (B) CD4 internalization is accelerated by NA7-GFP fusion protein. The percent fraction of CD4 molecules internalized in Jurkat T cells expressing NA7-GFP or GFP, determined as described in Materials and methods, is shown as a function of time. (C) Immunoblot analysis of NA7 and NA7-GFP expression. Aliquots of cytoplasmic extracts prepared from cells transfected with 17, 9.0, 3.0, 1.0 or 0.3 µg NA7 Nef or NA7-GFP expression vectors or, as a control, with 17 µg control empty vector (M), containing 40 µg protein, were immunoblotted with rabbit α -Nef serum and the immune complexes revealed by ECL.

previously for native Nef protein (Aiken *et al.*, 1994). Expression of NA7-GFP resulted in a 5- to 10-fold acceleration of the rate of CD4 internalization over that seen with cells expressing GFP protein alone (Figure 1B, compare NA7-GFP and GFP). Immunoblot analysis of cytoplasmic extracts prepared from transiently transfected cells with rabbit α -Nef serum revealed similar steady-state expression levels of NA7 and NA7-GFP proteins (Figure 1C, compare lanes 1–5 and 7–11). Moreover, transfection with the NA7-GFP expression vector did not result in any detectable expression of wild-type 27 kDa NA7 protein. Therefore, the properties required for down-regulation of surface CD4 by NA7 Nef are retained in the NA7-GFP fusion protein.

The NA7-GFP fusion protein co-localizes with the β -subunit of the adaptor protein complex at the cell periphery

To determine the subcellular localization of Nef, NA7-GFP protein was transiently expressed in CD4⁺ JJK T cells. As shown in Figure 2A, inspection of these cells by fluorescence microscopy revealed a punctate pattern of NA7-GFP fluorescence in peripheral sections of the cell (panel 2) and along the cell margin in equatorial cross-sections (panel 3). The NA7-GFP fluorescence was also consistently found in association with a large diffuse structure in the interior of JJK T cells (panel 3). In contrast to the well-defined, discrete pattern produced by NA7-GFP fusion protein, expression of the GFP protein alone produced a diffuse stain which decreased in intensity towards the cell margin (panel 1).

Using immunofluorescence staining the observed patterns of NA7-GFP fluorescence were shown to co-localize with the β -adapin subunits of adaptor complexes. The AP-2 and AP-1 adaptor complexes are largely restricted to clathrin-coated regions of the plasma membrane and membranes of the trans-Golgi network (Marks *et al.*, 1997; Robinson, 1997). As shown in Figure 2A, indirect immunofluorescence with the monoclonal antibody (mAb) 100/1, specific for β 1 and β 2 adaptins (Ahle *et al.*, 1988), revealed a staining pattern similar to that observed with NA7-GFP fusion protein (see panels 8 and 9). The two patterns show a high degree of co-localization of NA7-GFP fusion protein with β -adapin along the cell margin (compare panel 2 with 8 and 3 with 9). The two patterns also overlapped to a large extent in the perinuclear region of the cell, but this overlap appears less complete than that at the cell margin. To test whether the intracellular staining observed with NA7-GFP fusion protein reflects its co-localization with the AP-1 adaptor in the trans-Golgi network, immunofluorescent staining with a mAb specific for γ -adapin, a subunit of the AP-1 complex (100/3; Ahle *et al.*, 1988) was performed. As shown in Figure 2B, staining with mAb 100/3 revealed a pattern that overlaps with part of the pattern produced by NA7-GFP fusion protein. However, it can be clearly seen from overlay of the NA7-GFP and γ -adapin fluorescence patterns shown in panel 2 that NA7-GFP is also found in an additional, γ -adapin-negative area in the perinuclear region. The partial overlap of NA7-GFP and β - or γ -adapin patterns in the perinuclear region of the cell suggests that the three proteins mark distinct, but

Fig. 2. NA7-GFP fusion protein co-localizes with the β -adaptin, but not with the γ -adaptin subunit of adaptor complexes. (A) JJK T cells transfected with 1 μ g plasmids expressing GFP (panels 1, 4 and 7) or NA7-GFP (panels 2, 3, 5, 6, 8 and 9) were fixed and β_1 and β_2 adaptins detected with mAb 100/1 and visualized by indirect immunofluorescence (panels 7–9). GFP was revealed by direct fluorescence (panels 1–3). The overlays of GFP and β -adaptin images were produced using Oncor imaging software (panels 4–6). (B) Localization of NA7-GFP and γ -adaptin was performed with mAb 100/3 as described above.

possibly overlapping, subcompartments of the Golgi. While the exact relationship between NA7-GFP, β -adaptin and γ -adaptin in the Golgi area remains to be clarified,

our data indicate that Nef co-localizes with β -adaptin-containing AP-2 adaptor complexes at the plasma membrane.

Fig. 3. NA7-GFP fusion protein co-localizes with β -adaptin and with clathrin in REF52 fibroblasts. **(A)** REF52 fibroblasts transiently expressing GFP (panels 1, 5 and 9), NA7-GFP (panels 2, 3, 6, 7, 10 and 11) or $7_{(177AAA)}G$ proteins (panels 4, 8 and 12) were fixed and β_1 and β_2 adaptins were detected with mAb 100/1 and visualized by indirect immunofluorescence (panels 9–12). GFP was revealed by direct fluorescence (panels 1–4). The overlays of GFP and β -adaptin images were produced using Oncor imaging software (panels 5–8). Panels 3, 7 and 11 are magnifications of the boxed regions in panels 2, 6 and 10 respectively. Arrows indicate a subset of sites where NA7-GFP and β -adaptin patterns co-localize. **(B)** REF52 fibroblasts transfected with 1 μ g plasmid expressing NA7-GFP were fixed and clathrin heavy chain detected with mAb CHC5.9 and visualized by indirect immunofluorescence. Panels 4–6 are magnifications of the boxed regions in panels 1–3 respectively. Overlays of GFP and clathrin images were produced as described. Arrows indicate a subset of sites where NA7-GFP and clathrin patterns co-localize.

Co-localization of NA7-GFP with components of the clathrin coat does not require expression of CD4 molecules

To assess whether T cell-specific factors are required for the ability of NA7-GFP chimera to co-localize with

adaptor complex subunits, experiments in a rat embryo fibroblast cell line (REF52) were performed. As shown in Figure 3A, in REF52 cells the NA7-GFP chimera gave a perinuclear staining pattern with punctate labeling extending towards the cell periphery (panel 2). A very

Fig. 4. NA7-GFP co-localizes with CD4 at the plasma membrane. J.CaM1 cells co-transfected with 5 μ g plasmid expressing wild-type CD4 together with 1 μ g plasmids expressing GFP (panels 1, 4 and 7) or NA7-GFP (panels 2, 5 and 8) or with 5 μ g plasmid expressing a mutant protein CD4. Δ 402 that lacks most of the cytoplasmic domain together with 1 μ g NA7-GFP (panels 3, 6 and 9) were reacted with α -CD4 mAb 13B8.2 conjugated to PE-Cy5 and CD4 distribution was visualized by indirect immunofluorescence (panels 7–9). GFP and NA7-GFP were detected by direct fluorescence (panels 1–3). Overlays of GFP and CD4 images are shown in panels 4–6. Note that in contrast to JJK T cells, there is little perinuclear NA7-GFP and β -adaptin stain in JCaM1 cells (see panels 4–6; M.Greenberg, unpublished data).

similar pattern was produced by the β -adaptin-specific mAb 100/1 (panel 10). Close examination of the NA7-GFP and β -adaptin patterns in the peripheral regions of the cell revealed a large extent of co-localization (compare panels 3, 7 and 11 in Figure 3A). As a negative control in this experiment GFP and a mutant 7_(177AAA).G chimera, which do not co-localize with β -adaptins (see below), were used (panels 1, 5 and 9 and 4, 8 and 12 respectively).

To test whether NA7-GFP co-localizes with clathrin, another component of coats containing AP-1 and AP-2 adaptor protein complexes, co-localization experiments using mAb CHC5.9, which recognizes the α -heavy chain of clathrin, were performed. These experiments were performed in REF52 cells, rather than in T cells, because of the improved definition of fluorescence patterns. As shown in Figure 3B, the general patterns produced by NA7-GFP and by α -clathrin mAb CHC5.9 were similar (compare panels 1 and 3). Again, analysis of the magnified images demonstrated co-localization of the two patterns (compare panels 4 and 6). These observations suggest that Nef interacts, either directly or indirectly, with a component(s) of membrane structures containing AP-2 and clathrin and that these interactions occur independently of expression of CD4 or other T cell-specific factors.

Nef co-localizes with CD4 at the plasma membrane

To address the possibility that Nef interacts with CD4 molecules at the plasma membrane, we asked whether NA7-GFP protein co-localizes with CD4 expressed at the cell surface. Since the level of CD4 expression on the

surface of JJK T cells transiently expressing NA7-GFP was too low to permit CD4 detection by fluorescence microscopy (data not shown), experiments in CD4-negative J.CaM1 T cells (Goldsmith and Weiss, 1987) transiently transfected with CD4 and NA7-GFP expression plasmids or, as a control, with CD4 and GFP expression plasmids were performed. As shown in Figure 4, staining of live J.CaM1 T cells transiently expressing CD4 with an α -CD4 mAb (clone 13B8.2) under conditions that block endocytosis revealed a uniform distribution of CD4 at the cell margin (see panel 7). In contrast, in cells co-expressing CD4 and NA7-GFP CD4 was found redistributed in a distinct punctate pattern at the cell margin (panel 8). This punctate pattern co-localized with that for NA7-GFP (panel 5). Finally, cell surface expression of a mutant protein CD4. Δ 402, lacking most of the cytoplasmic domain and unresponsive to Nef-induced down-regulation from the cell surface (Salghetti *et al.*, 1995), was not affected by NA7-GFP protein (panel 9). Co-localization of CD4 at the cell surface with NA7-GFP and of NA7-GFP with β -adaptin at the cell margin together suggests that Nef redistributes CD4 to AP-2-containing coats at the plasma membrane.

Mutations in Nef that disrupt CD4 down-regulation differentially affect subcellular localization of NA7-GFP fusion protein

Previous genetic analysis found that the ability of Nef to down-regulate CD4 surface expression was selectively abolished by mutations in both the N- and C-terminal disordered domains of the Nef molecule (Iafrate *et al.*,

Fig. 5. Mutations in disordered regions of Nef disrupt CD4 down-regulation by NA7-GFP fusion proteins. Flow cytometry analysis of CD4 down-regulation by mutant 7.G proteins. JJK Jurkat T cells were transfected with 20 μ g vectors directing expression of NA7 Nef protein or its mutant versions fused to GFP, followed by two color flow cytometric analysis of CD4 and GFP expression. CD4 expression on GFP-positive cells is shown on the ordinate as peak channel number of CD4 fluorescence. The relative GFP fluorescence is shown on the abscissa. The effects of mutations in the disordered regions of Nef and of those in the SH3 binding surface in the Nef core are shown in the upper and lower panels respectively. The nomenclature of the mutant Nef proteins and of the corresponding mutations is defined in Figure 7.

1997), but not by those disrupting the SH3 binding surface in the structured Nef core (Saksela *et al.*, 1995). To further address the nature of Nef functions disrupted by these mutations, a panel of mutant NA7 Nef proteins was expressed as GFP fusions. Their effect on surface CD4 was tested in control experiments in JJK T cells. As expected, mutations in the disordered regions of Nef that were shown previously to block CD4 down-regulation by the NA7 Nef protein (Iafrate *et al.*, 1997) also disrupted this function in the context of the Nef-GFP fusion proteins (Figure 5, top panel; see Figure 7 for the definition of mutant Nef-GFP fusion proteins). As expected, mutations in the Nef core that did not have a significant effect on the ability of NA7 Nef protein to down-regulate CD4 expression had only a minor effect on function of NA7-GFP fusion protein (Figure 5, bottom panel). These observations again confirm that the NA7-GFP fusion protein faithfully retains the properties of wild-type NA7 Nef.

Using fluorescence microscopy we assessed the effect of these loss-of-function mutations in NA7-GFP Nef on subcellular localization of NA7-GFP and on co-localization of NA7-GFP with β -adaptins. As shown in Figure 6A, mutant Nef-GFP fusions that were unable to down-regulate surface CD4 fell into three distinct classes

on the basis of the staining pattern. The first class of mutants, represented by the 7(∇ HA).G protein, which contains an influenza hemagglutinin epitope (HA) insertion immediately following the initiator methionine in Nef, did not show the punctate pattern of NA7-GFP fluorescence along the cell margin. Instead, it produced a diffuse cytoplasmic staining pattern similar to that seen with GFP protein alone (Figure 6A, compare panel 3 with 1 and 2). The second class of mutants, represented by 7($_{29R,36G}$).G, which contains a double amino acid substitution E29R,D36G in the central part of the N-terminal disordered domain of the NA7 molecule, co-localized with β -adaptins at the cell margin, similar to wild-type NA7-GFP protein [7($_{29R,36G}$).G; compare panels 4 and 12 with 2 and 10]. The third class is represented by 7($_{56D}$).G, which contains the substitution D56A, and by 7($_{177AAA}$).G, containing a triple alanine substitution for E177, R178 and E179. These substitutions in the N- and C-terminal disordered domains of Nef severely disrupted the punctate pattern of GFP fluorescence along the cell periphery, but, in contrast to mutant 7(∇ HA).G of the first class, both 7($_{56D}$).G and 7($_{177AAA}$).G still appeared to stain the cell margin (compare panels 5 and 8 with 3). As shown in Figure 6B, steady-state expression of 7(∇ HA).G, 7($_{56D}$).G, 7($_{174K}$).G and 7($_{177AAA}$).G was similar to that for NA7-GFP Nef. With each of these mutant proteins one predominant band was detected by α -Nef and α -GFP sera. A slight variation in apparent molecular weight seen with some mutant proteins is likely to reflect conformational changes induced by the mutations and not degradation products (Mariani and Skowronski, 1993). While minor bands that migrated with GFP and slightly slower than GFP (see α -GFP panel) could result from degradation of 7.G proteins, these two bands were also found with NA7-GFP and 7($_{29R,36G}$).G (Figure 6B, bottom panel, lanes 7 and 9), which co-localize with β -adaptins. Therefore, these minor bands could not account for the different co-localization phenotypes observed for the 7($_{56D}$).G, 7($_{174K}$).G and 7($_{177AAA}$).G proteins. NA7-GFP proteins with mutations disrupting the SH3 domain binding interface in the Nef core [7($_{72A,75A}$).G and 7($_{86A}$).G] displayed patterns that were indistinguishable from those obtained with wild-type NA7-GFP fusion protein (compare panels 6 and 7 with 2). We conclude that several elements within the disordered domains of Nef cooperate to co-localize Nef with β -adaptins at the cell margin and that interactions at these elements are required for the ability of Nef to down-regulate surface CD4.

Several elements within the disordered regions of NA7 Nef are required for down-regulation of surface CD4

To further define the elements in the disordered domains of Nef that are essential for down-regulation of CD4 and their roles in this process, we analyzed a panel of chimeric NA7-GFP proteins bearing internal deletions in the NA7 moiety. Since changes at the N-terminal end of the NA7 molecule, arising either from HA epitope insertion at position 2 or from fusing the N-terminus of Nef to the C-terminal end of GFP, disrupted localization of the chimeric protein to the cell margin [see 7(∇ HA).G in Figure 6A and GFP-NA7 in Figure 8A], we tested whether the N-terminal Nef sequences can target GFP to the cell margin. As shown in Figure 8A, expression of a chimeric

protein comprising amino acid residues 1–10 of Nef fused to GFP in JJK T cells gave rise to a uniformly fluorescent band at the cell margin, but no peripheral punctate stain [$7_{(1-10)}\cdot G$, panel 3]. In addition, an intense fluorescence was detected in the perinuclear region, similar to that seen with the NA7–GFP fusion (compare panel 3 with 1). These observations are consistent with the possibility that the extreme N-terminal region of Nef is sufficient to direct Nef to cellular membranes.

Deletion of residues 11–41 did not have an appreciable effect on subcellular localization of the $7_{(\Delta 11-41)}\cdot G$ protein, yet it disrupted its ability to down-regulate surface CD4 (Figure 8A, panel 4, and Figure 8B). Immunoblot analysis of cytoplasmic extracts prepared from transiently transfected cells with α -Nef and α -GFP sera revealed that

Fig. 6. Mutations in Nef that disrupt CD4 down-regulation alter subcellular localization of the NA7–GFP fusion proteins. (A) JJK T cells were transfected with 1 μ g plasmids expressing mutant NA7 proteins fused to GFP (panels 3–8, 11–16 and 19–24) or control plasmids expressing the NA7–GFP fusion protein (panels 2, 10 and 18) or GFP protein alone (panels 1, 9 and 17). GFP was detected by direct fluorescence (panels 1–8) and β -adaptn was detected by indirect immunofluorescence (panels 17–24) with mAb 100/1 (Ahle *et al.*, 1988). The overlays of GFP and of β -adaptn images (panels 9–16) were produced using Oncor imaging software. Fragments of untransfected cells that do not express Nef–GFP fusion proteins can be seen next to the positively transfected cells in panels 23 and 24. Mutations in 7.G proteins are defined in Figure 7. (B) Immunoblot analysis of mutant 7.G proteins. Aliquots of cytoplasmic extracts prepared from JJK T cells transfected with 10 μ g plasmids encoding mutant 7.G proteins (lanes 8–12) and containing 10 μ g were immunoblotted with rabbit α -Nef serum (α -Nef, upper panel) or with α -GFP serum (α -GFP, lower panel). Serial 2-fold dilutions of extracts prepared from cells similarly transfected with NA7–GFP (lanes 1–5 in upper panel; amounts of extract relative to those in lanes 8–12 are indicated above the lanes) or GFP (lanes 1–5, lower panel) were used as standards for quantitation. Mock, extract (40 μ g) from cells transfected with a control empty vector.

steady-state expression of $7_{(\Delta 11-41)}\cdot G$ was similar to that for NA7–GFP (Figure 8D, compare lanes 8 and 10 with 1–5). A larger deletion spanning residues 11–66 of Nef in $7_{(\Delta 11-66)}\cdot G$ resulted in ~4- to 8-fold lower steady-state expression of the fusion protein. Thus, deleting residues 11–66 might have destabilized the fusion protein (Figure 8D, compare lanes 9 and 10 with lanes 1–5). Nevertheless, as shown in Figure 8A, $7_{(\Delta 11-66)}\cdot G$ produced an apparent punctate pattern at the cell margin (panel 5). We conclude that Nef sequences located distal to residues 11–66 are important for localization of Nef to discrete structures at the cell margin. This is consistent with observations from point mutations indicating that residues 174–179 are important for co-localization of Nef with β -adaptns at the cell margin (see Figure 6A, panel 8, and Figure 7).

Mutant	Mutation	Location	CD4 downregulation		-----Subcellular localization-----	
			NA7	NA7.GFP	Punctate (β -adaptins)	Cell margin
GFP			-	-	-	-
NA7.GFP	Nef(wt)-GFP		+++	+++	+++	+
7(2VHA)-G	2VHA		-	-	-	-
7(29R,36G)-G	G29R,D36G		-	-	+++	+
7(56D)-G	A56D		-	-	+	+
7(57AA)-G	W57A,L58A		-	-	-	++
7(57AAA)-G	W57A,L58A,E59A		-	-	++	++
7(72A,75A)-G	P72A,P75A		++	++	+++	+
7(86A)-G	D86A		+++	+++	+++	+
7(174K)-G	E174K		-	-	-	++
7(177AAA)-G	E177A,R178A,E179A		-	-	-	++

Fig. 7. Summary of point mutations in NA7 Nef protein and of their effects on CD4 down-regulation and on subcellular localization of NA7-GFP proteins. The nomenclature of the mutant Nef proteins and of the corresponding mutations is defined on the left side of the panel. The locations of the mutations, shown as black lollipops, in relation to the major structural features of the Nef protein are shown in the center of the panel. The disordered regions of HIV-1 Nef spanning amino acids 1–67 and 149–178 are represented by a line. The structured regions of the HIV-1 Nef core spanning residues 68–148 and 179–206 are represented by open bars (Grzesiek *et al.*, 1996a; Lee *et al.*, 1996). The N-terminal myristoylation in Nef (I) is shown. The GFP moiety is represented by a gray ellipsoid. The relative ability of the wild-type and mutant Nef proteins to down-regulate surface CD4 expression, derived from the data shown in Figure 5, and their subcellular localization shown in part in Figure 6A are summarized on the right side of the panel.

Discussion

We used an HIV-1 Nef-GFP fusion protein to study the functions of Nef required for down-regulation of CD4 surface expression in the human CD4⁺ Jurkat T cell line. Several lines of evidence from our studies support a model whereby NA7-GFP Nef protein interacts, either directly or indirectly, with CD4 and with clathrin coats containing AP-2 adaptor complexes at the plasma membrane. First, Nef co-localizes with known components of clathrin coats, such as the β -subunit of the adaptor protein complex and with clathrin at the cell margin. The cell margin corresponds to the plasma membrane, where AP-2-containing coats are commonly found (Marks *et al.*, 1997; Robinson, 1997). Second, we found that expression of NA7-GFP results in redistribution of CD4 to punctate structures at the cell margin, where it co-localizes with NA7-GFP protein. This observation demonstrates the physical proximity of CD4 at the plasma membrane to NA7-GFP Nef and, taken together with the previous observation, suggests that Nef recruits CD4 to AP-2-containing structures at the plasma membrane. Third, evidence from CD4-negative cell lines such as J.CaM1 T cells (data not shown) and REF52 fibroblasts indicates that NA7-GFP co-localization with β -adaptin does not require expression of CD4. Thus, a molecule(s) other than CD4 localizes NA7-GFP to the AP-2-containing coats at the plasma membrane. The conclusion that Nef co-localizes with components of the clathrin coat at the plasma membrane is consistent with data from another class of chimeric proteins in which the cytoplasmic domain of CD4 was replaced with Nef. The CD4-Nef chimeric molecule was frequently found in association with the coated pit regions of the plasma membrane (Mangasarian *et al.*, 1997). The genetic evidence from our studies correlates NA7-GFP and adaptor protein complex co-localization at the plasma membrane with the ability of Nef to down-regulate CD4 expression from the cell surface. Our observations support the possibility that Nef induces CD4 endocytosis via a clathrin-dependent pathway.

The NA7-GFP fusion protein and β -adaptins were also localized in a perinuclear compartment. This compartment

was not visualized following internalization of CD4 molecules stained at the cell surface with fluorescent mAbs nor following uptake of dextran and lucifer yellow tracers, suggesting that it is not an early endocytic compartment (M.Greenberg and J.Skowronski, unpublished data). Rather, based on a partial overlap of NA7-GFP, β -adaptin and γ -adaptin staining patterns and on known locations of β - and γ -adaptin-containing coats, this compartment is likely to be part of the Golgi network (Ahle *et al.*, 1988; Robinson, 1997). A partial overlap of the perinuclear NA7-GFP and adaptin patterns in the Golgi area raises the possibility that NA7-GFP may interact with AP-1 and/or AP-2 adaptor complexes at some of these locations, but the significance and specificity of this co-localization is not clear because of apparent differences in the NA7-GFP and β - and γ -adaptin patterns.

The ability of Nef to down-regulate CD4 expression from the cell surface was previously mapped to the N- and C-terminal disordered regions of the Nef molecule (Iafrate *et al.*, 1997). Our current genetic and functional studies indicate that these regions are involved in several molecular interactions that underlie the ability of Nef to down-regulate surface CD4. Two of these interactions, one mapping to the N-terminal 10 amino acid residues of Nef and the other involving residues 174–179 in the C-terminal disordered region of Nef, are required for co-localization of Nef with β -adaptins at the cell margin. Another interaction, which maps to the center of the N-terminal disordered region and is disrupted by mutating residues 29 and 36, does not significantly affect co-localization of Nef with β -adaptins. Thus, this latter interaction could be involved in recruitment of CD4 to coats containing AP-2 complexes or in promoting other aspects required for internalization of the CD4 molecule.

The co-localization of Nef with β -adaptins requires two functions that map to different regions within the Nef molecule. One such function is the ability of Nef to associate with cellular membranes. This is evident from the observation that mutations predicted to disrupt the N-terminal myristoylation signal in Nef disrupted co-localization of NA7-GFP protein with β -adaptins [see

Fig. 8. Deletion analysis of Nef sequences required for a punctate localization pattern. (A) JJK Jurkat T cells were transfected with 20 μ g plasmids expressing various chimeric NA7-GFP proteins or with control plasmids and the intracellular localization of mutant NA7-GFP protein was detected by fluorescence microscopy. (B) The effect of chimeric Nef proteins on CD4 expression was analyzed by flow cytometry as described in the legend to Figure 5. (C) A definition of mutants and their properties is shown. The positions of the previous mutations are indicated for comparison above the NA7-GFP diagram. (D) Immunoblot analysis of mutant 7.G proteins is shown. Aliquots of cytoplasmic extracts prepared from JJK T cells transfected with 10 μ g plasmids encoding mutant 7.G proteins (lanes 7–10) and containing 40 μ g were immunoblotted with rabbit α -Nef serum (α -Nef, upper panel) or with α -GFP serum (α -GFP, lower panel). Serial 2-fold dilutions of extracts prepared from cells similarly transfected with NA7 (40–2.5 μ g, lanes 1–5 in upper panel) or GFP (lanes 1–5, lower panel) were used as standards for quantitation. Mock, extract from cells transfected with a control empty vector (lane 7).

7_(2VHA).G in Figure 6A and GFP-NA7 in Figure 8A respectively] and from the observation that amino acids 1–10 of Nef contain a signal that directs GFP to the cell margin (Figure 8A, panel 3). The other function involved in this process maps to more distally located elements in

the Nef molecule. Specifically, mutations in the distal part of the N-terminal and in the internal disordered region disrupted co-localization of NA7-GFP Nef with β -adaptins, but did not affect localization of NA7-GFP to the cell margin [see 7_(56D).G, 7_(57AA).G, 7_(174K).G and

7_(177AAA)-G in Figure 6A and Figure 7]. These data support a model whereby the N-terminus of Nef and more distant elements in the Nef molecule cooperate to direct the NA7-GFP molecule to membrane structures containing the AP-2 coat.

The distal part of the N-terminal disordered region is involved in two interactions. One interaction is with plasma membrane structures containing AP-2 adaptor complexes and is disrupted by mutation D56A or WL57AA. The other interaction is required for CD4 down-regulation, but not for co-localization with the AP-2 adaptor protein complex, and is disrupted by mutation WLE57AAA. Results from a recent NMR study of interactions between Nef and CD4 cytoplasmic domain peptides suggested that residues W57, L58 and E59 in Nef are involved in direct contact between Nef and the CD4 cytoplasmic domain (Grzesiek *et al.*, 1996b). Our observation that mutating these residues disrupts CD4 down-regulation by Nef is consistent with this possibility. However, the different localization phenotypes of the WL57AA and WLE57AAA mutations argue that these residues may be important for folding or stability of Nef surfaces that have roles in a more complex set of interactions involving both CD4- and the AP-2 adaptor-containing structures.

Nef may have additional roles at the plasma membrane in promoting endocytosis of CD4 and other membrane proteins, such as MHC class I molecules (Schwartz *et al.*, 1996). For example, Nef could modulate aspects of morphogenesis of the AP-2-containing clathrin coat. Interestingly, T cells expressing HIV-1 and SIV Nef proteins show increased amounts of vesicular structures bearing endosomal and lysosomal markers (Sandfridson *et al.*, 1997). It should be noted that many aspects of the viral life cycle, including assembly and release of viral particles, involve interactions between additional viral gene products, such as Env and Gag, with the cellular sorting machinery (Marsh *et al.*, 1997). Thus interaction of Nef, a virion component (Pandori *et al.*, 1996), with clathrin-coated structures may also be relevant to assembly of viral particles and/or to viral entry and may have additional important roles in immunodeficiency virus pathogenesis.

Materials and methods

Plasmid construction

The NA7 Nef protein is encoded by a natural HIV-1 *nef* allele that has been described previously (Mariani and Skowronski, 1993). Oligonucleotide-directed site-specific mutagenesis was performed as previously described (Mariani and Skowronski, 1993; Iafrate *et al.*, 1997). The mutated *nef* sequences were verified by DNA sequencing and subcloned between the *Xba*I and *Bam*HI sites of a T cell-specific pCD3- β expression vector as previously described (Skowronski *et al.*, 1993). Genes directing expression of the fusion NA7-GFP and 7.G proteins were constructed using PCR and standard subcloning techniques (Sambrook *et al.*, 1989). In the NA7-GFP fusion protein the initial methionine of GFPsg25 (Palm *et al.*, 1997; G.Pavakis, unpublished data) was mutated to lysine and the two peptides linked by insertion of a GAGAS pentapeptide.

Cell lines and DNA transfections

Jurkat T cells (JJK) expressing high levels of human CD4, provided by Dan R.Littman, and the J.CaM1 subline of Jurkat T cells, provided by Mark A.Goldsmith (J.CaM1; Goldsmith and Weiss, 1987) were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, pH 7.4, and 10% fetal bovine serum (FBS) and the

cultures were diluted 1:10 to 1:20 every 3–4 days. REF52 rat embryo fibroblasts were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% FBS. Cells from exponentially growing cultures were transfected by electroporation as described previously (Mariani and Skowronski, 1993; Iafrate *et al.*, 1997). Briefly, cells were electroporated at 200 V and 960 μ F with a total of 10–20 μ g DNA containing varying amounts of the appropriate expression vectors and 3 μ g CMV CD20 expression plasmid, for use as a marker of transfected cells in flow cytometry experiments. The transfected cells were cultured for an additional 15–36 h prior to flow cytometric and/or microscopic analyses of CD4 and CD20 reporter levels and/or CD4 and GFP expression.

Immunoblot analysis

Cytoplasmic extracts from transiently transfected cells were prepared as described previously (Iafrate *et al.*, 1997). Immunoblot analysis of expression of Nef and the Nef fusion proteins was performed as described previously using affinity-purified polyclonal α -Nef (Skowronski *et al.*, 1993) or α -GFP (G.Pavakis, unpublished data) raised in rabbits and detected with the ECL system using the manufacturer's recommended conditions (Amersham).

Flow cytometry analysis

The effect of Nef on surface expression of CD4 in transiently transfected cells was analyzed on an Epics-Elite flow cytometer as described previously (Mariani and Skowronski, 1993; Iafrate *et al.*, 1997). Briefly, aliquots of 2×10^5 cells were suspended in phosphate-buffered saline (PBS) containing 1% FBS and 0.1% sodium azide (PBS-FA) and stained with saturating amounts of phycoerythrin-conjugated mAb Leu3A, specific for CD4 (Becton and Dickinson), or, in CD20-transfected cells expressing Nef proteins not fused to GFP in combination with PerCP-conjugated mAb Leu-16, specific for CD20 (Becton and Dickinson). For dose-response analysis the level of CD4 expression on the surface of CD20-positive cells, represented by peak channel number of green fluorescence, was measured as a function of the amount of Nef expression vector DNA used for transfections with non-GFP-tagged proteins (Skowronski and Mariani, 1995) or as a function of GFP fluorescence with GFP fusion proteins.

Endocytosis assay

Jurkat T cells were transfected with 20 μ g plasmids expressing NA7-GFP or GFP. Twenty-four hours after transfection aliquots of 10^7 cells were reacted with Leu3A α -CD4 mAb conjugated with phycoerythrin (Becton and Dickinson) for 30 min at 4°C in complete RPMI 1640 medium. The cells were then washed in ice-cold RPMI 1640 and aliquots of 10^6 were incubated at 37°C for the indicated amounts of time. Incubations were terminated by transferring the cells to an ice bath. Each sample was then divided into two aliquots and diluted 5-fold with PBS or with RPMI 1640 adjusted to pH 2 (acid wash). Total CD4 (surface + internalized) and internalized CD4 were determined by flow cytometry (Mangasarian *et al.*, 1997) for cells showing identical levels of GFP expression. The fraction of internalized CD4 was calculated as described previously (Mangasarian *et al.*, 1997).

Fluorescent microscopy analysis

Jurkat T cells, collected 15–36 h following transfection, were spun onto polylysine-coated coverslips. The coverslips were fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized in 0.1% NP40 in PBS. Cells were then incubated for 30 min in blocking solution (3% BSA, 0.1% NP40 in PBS) followed by a 1 h incubation with mAb 100/1, specific for β -adaptins, with 100/3 mAb, which recognizes γ -adaptin (Ahle *et al.*, 1988; both purchased from Sigma) or with mAb CHC5.9, which recognizes the α -heavy chain of clathrin (ICN), in blocking solution. Coverslips were washed in 0.1% NP40 in PBS, followed by a 30 min incubation with Cy-5-conjugated goat anti-mouse IgG antibody (Amersham), to detect β - and γ -adaptin, or Texas red-labeled goat anti-mouse IgM antibody (Southern Biotechnology Associates Inc.), to detect clathrin, and washed in PBS before mounting onto glass slides in glycerol-based mounting medium. For surface CD4 labeling $\sim 1 \times 10^6$ J.CaM1 cells were incubated with PE-Cy5-conjugated mAb specific for CD4 (Immunotech, clone 13B8.2), washed three times, spun onto polylysine-coated coverslips, fixed for 20 min in 3% paraformaldehyde and washed with PBS before mounting. The CD4 staining and all washes were performed at 4°C in the presence of 0.1% sodium azide, conditions which block CD4 endocytosis. Fluorescence microscopy images were taken with a Nikon Microphot-FXN microscope

equipped with a CCD camera and processed using Oncor Imaging software.

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References

- Ahle, S., Mann, A., Eichelsbacher, U. and Ungewickell, E. (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.*, **7**, 919–929.
- Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E. and Trono, D. (1994) Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell*, **76**, 853–864.
- Anderson, S., Shugars, D.C., Swannstrom, R. and Garcia, J.V. (1993) Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. *J. Virol.*, **67**, 4923–4931.
- Anderson, S.J., Lenburg, M., Landau, N.R. and Garcia, J.V. (1994) The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. *J. Virol.*, **68**, 3092–3101.
- Baur, A.S., Sawai, E.T., Dazin, P., Fantl, W.J., Cheng-Mayer, C. and Peterlin, B.M. (1994) HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity*, **1**, 373–384.
- Benson, R.E., Sanfridson, A., Ottinger, J.S., Doyle, C. and Cullen, B.R. (1993) Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.*, **177**, 1561–1566.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802–805.
- Collette, Y., Chang, H.L., Cerdan, C., Chambot, H., Algarte, M., Mawas, C., Imbert, J., Burny, A. and Olive, D. (1995) Specific Th1 cytokine downregulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression. *J. Immunol.*, **156**, 360–370.
- De, S.K. and Marsh, J.W. (1994) HIV-1 Nef inhibits a common activation pathway in NIH3T3 cells. *J. Biol. Chem.*, **269**, 6656–6660.
- Deacon, N.J. et al. (1995) Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science*, **270**, 988–991.
- Du, Z., Lang, S.M., Sasseville, V.G., Lackner, A.A., Ilyinskii, P.O., Daniel, M.D., Jung, J.U. and Desrosiers, R.C. (1995) Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell*, **82**, 665–674.
- Garcia, J.V. and Miller, A.D. (1991) Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature*, **350**, 508–511.
- Goldsmith, M.A. and Weiss, A. (1997) Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. *Proc. Natl Acad. Sci. USA*, **84**, 6879–6883.
- Graziani, A., Galimi, F., Medico, E., Cottone, E., Gramaglia, D., Boccaccio, C. and Comoglio, P.M. (1996) The HIV-1 Nef protein interferes with phosphatidylinositol 3-kinase activation. *J. Biol. Chem.*, **271**, 6590–6593.
- Grzesiek, S., Bax, A., Clore, G.M., Gronenborn, A.M., Hu, J.S., Kaufman, J., Palmer, I., Stahl, S.J. and Wingfield, P.T. (1996a) 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. *Nature Struct. Biol.*, **3**, 340–345.
- Grzesiek, S., Stahl, S.J., Wingfield, P.T. and Bax, A. (1996b) The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. *Biochemistry*, **35**, 10256–10261.
- Hua, J., Blair, W., Truant, R. and Cullen, B.R. (1997) Identifications of regions in HIV-1 Nef required for efficient downregulation of cell surface CD4. *Virology*, **231**, 231–238.
- Iafrate, A.J., Bronson, S. and Skowronski, J. (1997) Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling. *EMBO J.*, **16**, 673–684.
- Kestler, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D. and Desrosiers, R.C. (1991) Importance of the nef gene for maintenance of high virus load and development of AIDS. *Cell*, **65**, 651–662.
- Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L. and Desrosiers, R.C. (1995) Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *New Engl. J. Med.*, **332**, 228–232.
- Lee, C.-H., Saksela, K., Mirza, U.A., Chait, B.T. and Kuryan, J. (1996) Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell*, **85**, 931–942.
- Liu, L.X., Margottin, F., LeGall, S., Schwartz, O., Selig, L., Benarous, R. and Benichou, S. (1997) Binding of HIV-1 Nef to a novel thiolesterase enzyme correlates with Nef-mediated CD4 downregulation. *J. Biol. Chem.*, **272**, 13779–13785.
- Luria, S., Chambers, I. and Berg, P. (1991) Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. *Proc. Natl Acad. Sci. USA*, **88**, 5326–5330.
- Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpentier, J.-L. and Trono, D. (1997) The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. *Immunity*, **6**, 67–77.
- Mariani, R. and Skowronski, J. (1993) CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type I-infected individuals. *Proc. Natl Acad. Sci. USA*, **90**, 5549–5553.
- Mariani, R., Kirchhoff, F., Greenough, T.C., Sullivan, J.L., Desrosiers, R.C. and Skowronski, J. (1996) High frequency of defective nef alleles in a long-term survivor with nonprogressive HIV-1 infection. *J. Virol.*, **70**, 7752–7764.
- Marks, M.S., Ohno, H., Kirchhausen, T. and Bonifacio, J.S. (1997) Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores. *Trends Cell Biol.*, **7**, 124–128.
- Marsh, M. and Pelchen-Matthews, A. (1996) Endocytotic and exocytotic regulation of CD4. *Curr. Topics Microbiol. Immunol.*, **205**, 107–135.
- Marsh, M., Pelchen-Matthews, A. and Hoxie, J.A. (1997) Roles for endocytosis in lentiviral replication. *Trends Cell Biol.*, **7**, 1–4.
- Myers, G., Berzofsky, J.A., Rabson, A.B. and Smith, T.E. (1993) *Human Retroviruses and AIDS*. Los Alamos National Laboratory, Los Alamos, NM.
- Nunn, M.F. and Marsh, J.W. (1996) Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *J. Virol.*, **70**, 6157–6161.
- Ohno, H. et al. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science*, **269**, 1872–1875.
- Palm, G.J., Zolov, A., Gaitanaris, G.A., Strauber, R., Paulakis, G.N. and Woulawer, A. (1997) The structural basis for spectral variation in green fluorescent protein. *Nature Struct. Biol.*, **4**, 361–365.
- Pandori, M.W., Fitch, N.J., Craig, H.M., Richman, D.D., Spina, C.A. and Guatelli, J.C. (1996) Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. *J. Virol.*, **70**, 4283–4290.
- Pelchen-Matthews, A., Parsons, I.J. and Marsh, M. (1993) Phorbol ester-induced downregulation of CD4 is a multi-step process involving dissociation from p56^{lck}, increased association with clathrin-coated pits and altered endosomal sorting. *J. Exp. Med.*, **178**, 1209–1222.
- Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J. (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene*, **111**, 229–233.
- Rhee, S.S. and Marsh, J.V. (1994) Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J. Virol.*, **68**, 5156–5163.
- Robinson, M.S. (1997) Coats and vesicle budding. *Trends Cell Biol.*, **7**, 99–102.
- Saksela, K., Cheng, G. and Baltimore, D. (1995) Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J.*, **14**, 484–491.

- Salghetti,S., Mariani,R. and Skowronski,J. (1995) Human immunodeficiency virus type 1 Nef and p56^{lck} protein tyrosine kinase interact with a common element in CD4 cytoplasmic tail. *Proc. Natl Acad. Sci. USA*, **92**, 349–353.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanfridson,A., Cullen,B.R. and Doyle,C. (1994) The simian immunodeficiency virus Nef protein promotes degradation of CD4 in human T cells. *J. Biol. Chem.*, **269**, 3917–3920.
- Sanfridson,A., Hestler,S. and Doyle,C. (1997) Nef proteins encoded by human and simian immunodeficiency viruses induce the accumulation of endosomes and lysosomes in human T cells. *Proc. Natl Acad. Sci. USA*, **94**, 873–878.
- Sawai,E.T., Baur,A., Struble,H., Peterlin,B.M., Levy,J.A. and Cheng-Mayer,C. (1994) Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc. Natl Acad. Sci. USA*, **91**, 1539–1543.
- Schwartz,O., Riviere,Y., Heard,J.-M. and Danos,O. (1993) Reduced cell surface expression of processed human immunodeficiency type 1 envelope glycoprotein in the presence of Nef. *J. Virol.*, **67**, 3274–3280.
- Schwartz,O., Dautry-Varsat,A., Goud,B., Marechal,V., Subtil,A., Heard,J.-M. and Danos,O. (1995) Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes. *J. Virol.*, **69**, 528–533.
- Schwartz,O., Marechal,V., LeGall,S., Lemonnier,F. and Heard,J.M. (1996) Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nature Med.*, **2**, 338–342.
- Skowronski,J. and Mariani,R. (1995) Transient assay for Nef-induced down-regulation of CD4 antigen expression on the cell surface. In Karn,J. (ed.), *HIV-1: A Practical Approach*. IRL Press, Oxford, UK, pp. 231–242.
- Skowronski,J., Parks,D. and Mariani,R. (1993) Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. *EMBO J.*, **12**, 703–713.
- Sleckman,B.P., Shin,J., Igras,V.E., Collins,T.L. and Strominger,J.L. (1992) Disruption of the CD4-p56lck complex is required for rapid internalization of CD4. *Proc Natl Acad. Sci. USA*, **87**, 7566–7570.
- Traub,L.M. (1997) Clathrin-associated adaptor proteins—putting it all together. *Trends Cell Biol.*, **7**, 43–46.

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