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Abstract
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Reference

PMID : 10199403
Nef-Induced CD4 Degradation: A Diacidic-Based Motif in Nef Functions as a Lysosomal Targeting Signal through the Binding of β-COP in Endosomes

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Summary

The Nef protein of primate lentiviruses downregulates the cell surface expression of CD4 through a two-step process. First, Nef connects the cytoplasmic tail of CD4 with adaptor protein complexes (AP), thereby inducing the formation of CD4-specific clathrin-coated pits that rapidly endocytose the viral receptor. Second, Nef targets internalized CD4 molecules for degradation. Here we show that Nef accomplishes this second task by acting as a connector between CD4 and the β subunit of COPI coatomers in endosomes. A sequence encompassing a critical acidic dipeptide, located nearby but distinct from the AP-binding determinant of HIV-1 Nef, is responsible for β-COP recruitment and for routing to lysosomes. A novel class of endosomal sorting motif, based on acidic residues, is thus revealed, and β-COP is identified as its downstream partner.

Introduction

The Nef protein is an important virulence factor of primate lentiviruses. Studies in adult rhesus monkeys infected with simian immunodeficiency virus (SIV) demonstrate that Nef greatly potentiates viremia and precipitates disease (Kestler et al., 1991). In humans, HIV-1 strains deleted in nef can be isolated from individuals who behave as long-term nonprogressors of the infection (Deacon et al., 1995; Kirchhoff et al., 1995). Finally, studies in transgenic mice confirm that Nef, on its own, is a major determinant of pathogenicity (Hanna et al., 1998).

The biological phenomena underlying the role of Nef in disease induction are complex, but significant progress has been gained in dissecting their mechanisms (reviewed in Cullen, 1998; Emerman and Malim, 1998). HIV-1 Nef is a 206 amino acid-long cytoplasmic protein that associates with cell membranes through myristoylation (Niederman et al., 1993). Produced in abundance early in the viral life cycle, Nef downregulates CD4 (Guy et al., 1987; García and Miller, 1991) and class I major histocompatibility complex (MHC I) (Schwartz et al., 1996; Collins et al., 1998), stimulates the efficiency of HIV-1 proviral DNA synthesis (Aiken and Trono, 1995; Schwartz et al., 1995), and alters cellular activation pathways (Baur et al., 1994; Iafrate et al., 1997; Hanna et al., 1998). These effects are genetically distinguishable yet highly conserved.

CD4 is a type I integral membrane glycoprotein expressed on the surface of thymocytes, T helper lymphocytes, and cells of the monocyte/macrophage lineage (Maddon et al., 1986). Required for both the maturation and activation of T helper lymphocytes, CD4 stabilizes the interaction between the T cell receptor (TCR) on the surface of lymphocytes and the class II major histocompatibility complex (MHC II) on antigen-presenting cells. Furthermore, it recruits the p56Lck protein tyrosine kinase, bound to its cytoplasmic domain, to the vicinity of the TCR. As such, CD4 functions as a coactivator, because Lck is a major proximal effector of the T cell activation cascade (reviewed in Weiss and Littman, 1994).

CD4 also serves as the primary receptor for both HIV and SIV and is dramatically downregulated following infection. One important role of this downmodulation is to permit the subsequent release of fully infectious virions, as excessive levels of CD4 on the surface of virus-producing cells result in blocking the virion incorporation and function of the viral envelope (Lama et al., submitted). Two HIV-1 proteins, Nef and Vpu, counteract this inhibition by downmodulating the cell surface expression of CD4. Of the two, Nef exerts the earliest and most robust influence (Chen et al., 1996; Lama et al., submitted).

The study of Nef-induced CD4 downregulation has provided exciting insights into the mechanisms by which cells can modulate the expression of a surface molecule. Nef was demonstrated to trigger the accelerated endocytosis of CD4 by acting as a connector between the receptor and clathrin-coated pits (CCP). Two interactions govern this process. First, a cluster of residues in the N-terminal half of HIV-1 Nef recognizes a dileucine-based motif in the membrane-proximal portion of the CD4 cytoplasmic tail (Aiken et al., 1994; Grzesiek et al., 1996b). Second, Nef interacts with the adaptor protein complex (AP) of CCP, triggering the de novo formation of pits that are largely CD4 specific (Foti et al., 1997; Mangasarian et al., 1997; Le Gall et al., 1998; Piguet et al., 1998). Adaptor complexes are heterotetrameric structures that normally recruit clathrin to the cytoplasmic tail of receptors containing endocytosis signals (reviewed in Robinson, 1994). These are usually based either on tyrosine or on a leucine dipeptide (Letourneur and Klausner, 1992; Kirchhausen et al., 1997). An interaction between the Nef protein of primate lentiviruses and the medium chain of adaptor complexes was demonstrated both in the yeast two-hybrid system and in vitro with recombinant proteins (Le Gall et al., 1998; Piguet et al., 1998). For HIV-2 Nef and SIV Nef, the interaction is mediated by two adjacent tyrosine-based motifs in the N-terminal region of the viral proteins (Piguet et al., 1998). HIV-1 Nef does not harbor this sequence and instead recruits APs via a dileucine-based signal.
located in its C-terminal region (LL165) (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998). Nef mutants defective for AP binding are unable to accelerate CD4 internalization, either in trans or when fused to the extracellular and transmembrane domains of the receptor. This demonstrates that adaptor complexes are a major downstream partner of Nef for CD4 endocytosis.

Additional Nef-binding proteins might facilitate the interaction of Nef with adaptors, including the recently identified 54 kDa Nef-binding protein (NBP1) (Lu et al., 1998). This protein shares sequence similarities with the catalytic subunit of the vacuolar ATPase (V-ATPase) and is the mammalian homolog of the S. cerevisiae VMA13p protein, the catalytic component of the universal proton pump. NBP1 is also capable of interacting with AP-2 and is probably recruited into CCPs. Our analyses, however, indicate that Nef alleles mutated in residues reported to be critical for NBP1 binding are still active for accelerating endocytosis (Mangasarian et al., 1999).

Surprisingly, Nef mutants defective for AP binding conserve a significant ability to downmodulate the cell surface expression of CD4 (Piguet et al., 1998). This puzzling observation led to the discovery that the downregulation of CD4 by the Nef protein of primate lentiviruses is the result of two consecutive steps. Indeed, after triggering the rapid endocytosis of CD4, Nef prevents the cell surface recycling of the glycoprotein, which is instead targeted to a degradation pathway (Oldridge and Marsh, 1998; Piguet et al., 1998). Remarkably, the two actions of Nef are functionally and genetically separable, indicating that they are accomplished by distinct regions of the protein and most likely through interactions with different cellular partners (Piguet et al., 1998). Here we elucidate the mechanism of the second effect of Nef, as we reveal that the viral protein targets CD4 for lysosomal degradation by connecting the receptor with the β subunit of the COPI coatomer. Furthermore, we demonstrate that β-COP functions in this process as an endosomal sorting molecule that recognizes a diacidic-based lysosomal targeting signal in Nef.

Results

An Acidic Dipeptide in HIV-1 Nef Is Necessary for Preventing the Cell Surface Recycling of CD4

A series of HIV-1 Nef mutants impaired for CD4 downmodulation was previously identified. The first was a myristoylation-defective Nef derivative, which indicated that Nef membrane attachment is necessary for regulating CD4 (Aiken et al., 1994). The functional defect of a group of mutants resulted from a failure to bind CD4. This was in particular the case of derivatives with amino acid changes in the N-terminal CD4-binding domain of Nef (RD36AA, WL58AA) (Grzesiek et al., 1996b; Mangasarian et al., 1999). Another mutation (LL165AA) abrogated Nef-induced CD4 downregulation by preventing the capture of AP complexes by the viral protein (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998). One HIV-1 Nef mutant, however, appeared to owe its defective phenotype to a distinct mechanism. In this variant, a highly conserved acidic dipeptide (EE155) in a C-terminal disordered loop of the protein was replaced by two glutamine residues (Aiken et al., 1996). As previously reported, NefEE155QQ was only 25% as active as wild-type Nef at downregulating the steady-state levels of cell surface CD4 (Figure 1A). However, this mutant was fully active on CD4 endocytosis, as 6% of CD4 molecules initially present on the surface of wild-type Nef- and NefEE155QQ-expressing cells were internalized...
per minute during the first 5 min, versus 2% in control cells (Figure 1B).

This result suggested that Nef_{EE155QQ} might be selectively defective in its ability to decrease the cell surface recycling of CD4. A FACS-based CD4 assay was used to test this hypothesis, as described (Pelchen-Matthews et al., 1989; Piguet et al., 1998) (Figure 1C). In transfected 293T cells, about 45% of internalized CD4 recycled back to the cell surface within 10 min. In the presence of wild-type HIV-1 Nef, this fraction was reduced to about 10%, consistent with measurements previously obtained with SIV Nef (Piguet et al., 1998). At later time points, in both control and nef-expressing cells, the percentage of recycled CD4 increased, but wild-type Nef still had a marked negative effect. In striking contrast, more than 42% of endocytosed CD4 molecules rapidly returned to the surface of cells producing Nef_{EE155QQ}. Importantly, wild-type Nef affected neither the internalization nor the recycling rate of the transferrin receptor (not illustrated).

Chimeric integral membrane proteins that comprise the extracellular and transmembrane regions of CD4 with HIV-1 Nef as their cytoplasmic domain were then expressed in 293T cells by transient transfection. As previously described (Mangasarian et al., 1997), a fusion protein between CD4 and wild-type Nef (called 44Nef) induced steady-state cell surface levels that were at least 10-fold lower than those of CD4 (Figure 2A). The 44Nef_{EE155QQ} molecule exhibited an intermediate phenotype, as it was expressed around five times more efficiently on the cell surface than its wild-type Nef counterpart. Nevertheless, 44Nef and 44Nef_{EE155QQ} were internalized with similar kinetics, which far exceeded those of CD4 (Figure 2B). In contrast, while the 44Nef fusion protein was efficiently retained inside the cell after endocytosis, with only 10% recycling after 10 min, 44Nef_{EE155QQ} returned to the cell surface as effectively as CD4 (Figure 2C). Interestingly, the 44Nef_{LL165AA} variant, which undergoes slow rates of endocytosis because it fails to bind AP, exhibited a block in recycling similar to that of 44Nef and at steady state has surface levels comparable to those of 44Nef_{EE155QQ} (not illustrated). This parallels our prior observation that an SIV Nef derivative mutated in a tyrosine-based signal crucial for AP recognition fails to accelerate the internalization of CD4 yet still prevents its recycling (Piguet et al., 1998).

In order to confirm the role of the Nef diacidic motif in these events, fusion proteins comprising the extracellular and transmembrane domains of CD4 and the IL-2 receptor alpha subunit (TAC) cytoplasmic domain were created. TAC undergoes only minimal levels of endocytosis and is efficiently recycled (Radhakrishna and Donaldson, 1997). A sequence encompassing amino acids 151 to 165 of HIV-1 Nef, either wild type or mutated in the EE_{155} acidic dipeptide, was then added to the C terminus of the CD4-TAC chimera (Figure 3A). The resulting 44TAC-Nef_{151-165} and 44TAC-Nef_{151-165EE155QQ} molecules were internalized more rapidly than the parental 44TAC (not illustrated), reflecting the activity of the Nef LL_{165} dileucine-based endocytosis signal (Craig et al., 1998). While 44TAC-Nef_{151-165} was retained intracellularly after internalization, with less than 10% recycling after 10 min, both 44TAC-Nef_{151-165EE155QQ} and 44TAC rapidly returned to the cell surface following internalization at rates that exceeded 40% after 10 min (Figure 3B).

Figure 2. cis Effect of Wild-Type and EE_{155}-Mutated Nef in the Context of CD4-Nef Chimeras
(A) Steady-state surface levels of CD4 and CD4-Nef chimeras, evaluated by flow cytometry.
(B) Rates of endocytosis of CD4 and CD4-Nef chimeras, confirming that both the wild-type and the EE_{155} mutant Nef interact with the cellular internalization machinery.
(C) Recycling assay; the EE_{155} motif of Nef prevents the cell surface return of a CD4-Nef chimera.

Results are representative of three experiments. Error bars correspond to 1 standard deviation from the mean.
Figure 3. A 15 Amino Acid–Long Nef Sequence, Centered around the Critical EE155 Motif, Can Prevent the Postendocytic Recycling of a Chimeric Integral Membrane

(A) Schematic representation of the various constructs used in this experiment. The white boxes represent CD4-based domains, the black boxes the TAC cytoplasmic tail, and the shaded boxes Nef-derived sequences. The Nef151–165 amino acid sequence is represented underneath, in single-letter code. The EE155 acidic and LL165 leucine dipeptides are in bold characters.

(B) Recycling assay. 44TAC and 44TAC-Nef150–166(EE155QQ), but not 44TAC-Nef151–165, efficiently return to the cell surface after internalization.

The Diacidic-Based Motif of HIV-1 Nef Targets CD4 to Degradation in an Acidic Compartment

HIV-1 Nef was previously shown to induce CD4 degradation, both in trans and within the context of CD4– or CD8–Nef chimeras (Aiken et al., 1994; Rhee and Marsh, 1994; Mangasarian et al., 1997). To investigate a possible role for the EE155 diacidic motif in this effect, 293T cells transiently transfected with vectors expressing CD4, 44Nef, and 44NefEE155QQ were pulse labeled with 35S amino acids. Extracts harvested at different time points were immunoprecipitated with CD4-specific antibody, and the immune complexes were analyzed by gel electrophoresis and quantitative autoradiography (Figure 4A). The results indicated that the half-life of CD4 in this system was approximately 14 hr, while that of 44Nef was 6 hr, consistent with previous observations (Mangasarian et al., 1997). By comparison, 44NefEE155QQ had a half-life of about 11 hr, indicating that the EE155 diacidic-based motif of Nef is involved in accelerating the degradation of the CD4–Nef chimera.

Figure 4. The EE155 HIV-1 Nef Motif Is Important for Targeting CD4 to an Acidic Degradation Compartment

(A) Half-life of CD4 and CD4–Nef chimeras in transiently transfected 293T cells. After a 30 min pulse with 35S-labeled amino acids, cellular extracts normalized for luciferase activity (from a cotransfected reporter construct) were immunoprecipitated with a CD4-specific antibody. Immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography (shown here) and quantitated using a phospho-image.

(B) Cells expressing CD4 either alone or together with wild-type or EE155QQ Nef were surface labeled with a fluorescein-conjugated antibody. After incubation at 37°C to allow for internalization, the pH of CD4 vesicles was measured as described in Experimental Procedures. Top: mean pH of CD4 vesicles; number on top of bar indicates number of vesicles included in each analysis; error bars correspond to 1 standard deviation from the mean. Bottom: composite images of two representative cells integrating fluorescence and pH, scaled in pseudocolors. With wild-type Nef (upper cell), anti–CD4 antibody–containing vesicular structures exhibit the purple–to–blue coloration indicative of a pH around 5.5. The green color of
Delivery of pH-sensitive probes by receptor-mediated endocytosis has facilitated the study of the pH along the endocytic pathway, revealing that the acidity of the lumen increases from a pH of 6.0–6.5 in early and recycling endosomes to 5.0–5.9 in late endosomes and 5.0–5.5 in lysosomes (Mukherjee et al., 1997). Biochemical analyses previously indicated that Nef-induced CD4 degradation takes place in an acidic compartment, most likely the late endosome/lysosome (Aiken et al., 1994; Rhee and Marsh, 1994). Based on this dual premise, 293T cells expressing CD4 alone, or together with either Nef or NefEE155QQ, were incubated with a pH-sensitive FITC-conjugated CD4-specific antibody for 45 min at 37°C to allow for internalization. The pH of the subcellular compartment in which the internalized antibody resided was then evaluated by fluorescence microscopy as previously described (Demaurex et al., 1998) (Figure 5). In the presence of wild-type HIV-1 Nef, the mean pH value of CD4 vesicles was 5.6, consistent with the degradative compartment. In contrast, when CD4 was expressed either alone or in the presence of the EE155QQ Nef mutant, it concentrated in vesicles that had a mean pH value of 6.4 and 6.3, respectively. This is a strong indication that, in these latter two situations, the receptor failed to enter the late endosomal/lysosomal pathway.

The Nef Diacidic-Based Motif Functions as a Lysosomal Targeting Signal

To ascertain that the acidic dipeptide of Nef behaves as a lysosomal targeting signal, ultrastructural studies were performed. Briefly, 293T cells transiently transfected with CD4, 44Nef, or 44NefEE155QQ were incubated at 4°C with an anti-CD4 antibody followed by a secondary antibody coupled to colloidal gold. The cells were incubated for 10 min at 37°C to allow for internalization of the receptor-antibody complex, before fixation and processing for electron microscopy. Various structures containing gold particles were visualized and divided into subgroups according to their morphology (Figure 5). All three CD4 derivatives were localized in easily recognizable clathrin-coated structures, which were not examined further. Instead, we made use of the typical ultrastructural appearance of endosomes in the recycling and degradation pathways, respectively, to analyze the preferential routes followed by internalized CD4 derivatives. Indeed, recycling endosomes form networks of tubules with a small (around 60 nm) diameter (referred to as TVS for tubular-vesicular structures) (Figure 5, top) (Tooze and Hollinshead, 1991; Hopkins et al., 1994). In contrast, endosomal elements along the degradation pathway leading to the lysosomes, including the transport intermediates between early and late endosomes (endosomal carrier vesicles/multivesicular bodies, ECV/MVBs) and the late endosomes themselves, contain a characteristic accumulation of internal membranes within their lumen (called MVB/L for multivesicular bodies/lysosomes) (Figure 5, bottom) (Griffiths et al., 1988; Gruenberg et al., 1989; Futter et al., 1996; Geuze, 1998; Kobayashi et al., 1998). We also scored vacuolar structures in the cell periphery, often connected to the tubular network, as sorting early endosomes (called VS for vacuolar structures). Ten minutes after internalization, 44Nef exhibited a high propensity to accumulate in sorting endosomes and in endosomal elements of the degradation pathway: about 57% of 44Nef molecules resided in these structures, whereas only 40% were found in recycling elements. An inverse picture was recorded with CD4 and 44NefEE155QQ, which were much less frequently detected in endosomes of the degradation pathway and in sorting endosomes (21% and 18%, respectively) and were instead mainly (75%) associated with recycling endosomes.

HIV-1 Nef Lysosomal Targeting Function Correlates with its Ability to Bind β-COP

Nef triggers CD4 endocytosis by acting as a connector between the receptor and the adaptor complex of CCP. By analogy, it was tempting to postulate that the lysosomal targeting function of Nef also reflects an interaction with a specific downstream partner. However, this partner was unlikely to be the AP, because the Nef determinants required for triggering the internalization of CD4 or for inducing its degradation are distinct. One protein, the β-COP subunit of COPI coatomers, stood out as an attractive candidate for mediating the Nef-induced lysosomal sorting of CD4. Although COPI vesicles govern transport at early stages of the biosynthetic pathway (Orci et al., 1993; Pepperkok et al., 1993; Cosson and Letourneur, 1994; Letourneur et al., 1994), recent morphological and functional evidence suggests a role for a...
subset of β-COP-containing coatomers in the transport from early to late endosomes (Whitney et al., 1995; Aniento et al., 1996; Daro et al., 1997; Gu et al., 1997). Furthermore, β-COP was identified as a Nef-binding protein through a yeast two-hybrid screen, and the interaction between the two proteins was confirmed in vitro (Benichou et al., 1994).

To investigate a possible role of β-COP in Nef-induced lysosomal targeting, recombinant GST-Nef fusion proteins were purified from E. coli, coupled to glutathione beads, and used as baits to capture proteins from CHO cell lysates. GST-Nef-bound proteins were analyzed by Western blotting using antibodies against β-COP, δ-COP, and γ-adaptin (Figure 6A). Wild-type GST-Nef, but not the GST-NefEE155QQ variant, could recruit β-COP, indicating the essential role of the EE155 dipeptide in this process. Under the same conditions, Nef did not bind δ-COP. This is of interest because, while COP proteins associated with the biosynthetic pathway assemble in a complex of seven subunits including α, β, β’, δ1, γ, ε, and ε’, the δ and γ subunits are absent from the endosomal COP complex (Whitney et al., 1995). The interactions between Nef and adaptor complexes were also analyzed. Corroborating the results of the endocytosis assays, both GST-Nef and GST-NefEE155QQ could capture the α chain of the adaptor complex AP-2 (Figure 6A). The diacidic motif-dependent binding of GST-Nef with β-COP was also noted when using cytoplasmic fractions enriched in COPI coatomer (Aniento et al., 1996). In this case, the binding was strongly enhanced by the addition of COP-depleted cytosolic extracts, indicating a facilitating role for some soluble factor(s) (Figure 6B). This is reminiscent of the recently demonstrated requirement of ADP-ribosylation factor (ARF) family proteins and nucleotides for the efficient recruitment of coatomer on synthetic liposomes carrying the cytoplasmic tail of the putative transmembrane cargo receptor p23 (Bremser et al., 1999). Similar cofactors might promote the Nef-coatomer interaction.

In order to ascertain that a direct association between Nef and β-COP was taking place, binding assays were repeated with in vitro-translated β-COP (Figure 6C). In this system too, a specific interaction between the coatomer subunit and GST-Nef was detected, well above the background observed with GST and GST-NefEE155QQ. Interestingly, the LL165AA mutation did not affect the binding of Nef to β-COP, as predicted from our functional analyses indicating that the AP-binding leucine dipeptide is not required for the lysosomal targeting function of Nef. Also, the EE155-dependent binding of Nef to β-COP was demonstrated with a bacterially produced C-terminal fragment of β-COP (amino acids 476–925) and recombinant HIV-1 Nef (data not shown).

Finally, the role of COPI coatomers in Nef-induced CD4 lysosomal targeting was demonstrated through an additional functional assay. The ldld mutant CHO cell line bears a temperature-sensitive defect in the COPI subunit ε-COP. As a consequence, at the restrictive temperature, ldld cells display a defect in endosomal sorting and in transport from early to late endosomes, while internalization still proceeds normally (Daro et al., 1997; Gu et al., 1997). The effect of Nef on the routing of internalized CD4 molecules was therefore examined in

Figure 6. Diacidic-Dependent Binding of Nef to β-COP
(A) Cytoplasmic extracts from CHO cells were incubated with the indicated GST fusion proteins. After extensive washes, bound material was analyzed by Western blotting with antibodies against β- or ε-COP (top) or α-adaptin (bottom). GST-Nef and GST-NefEE155QQ, but not GST, can capture whole adaptor complexes, but β-COP recruitment requires the EE155 sequence. Quantitative measurements of Nef–β-COP interaction, based on four independent experiments, were as follows (±SEM): GST-Nef, 1.0 (arbitrary value); GST, 0.05 (±0.01); GST-NefEE155QQ, 0.09 (±0.03).
(B) Two hundred micrograms of a COP-enriched fraction from rat liver cytosol with or without 40 μg of a COP-depleted fraction was incubated in the presence of the indicated GST fusion proteins. After five washes, bound material was analyzed by Western blotting using antibodies against β-COP. GST-Nef could capture β-COP from a COP-enriched fraction, but the recruitment of β-COP was enhanced approximately 8-fold when a COP-depleted fraction was added in the reaction. Note that GST-Nef does not capture any detectable β-COP from a COP-depleted fraction alone. GST and GST-NefEE155QQ are unable to interact with β-COP in these conditions.
(C) GST-Nef and GST-NefEE155QQ, but not GST or GST-NefEE155QQ, can capture 35S-labeled in vitro-translated ε-COP. The experiments were performed as described in Experimental Procedures. Quantitative measurements of β-COP capture (based on four independent experiments, ±SEM): GST-Nef, 1.0 (arbitrary value); GST, 0.15 (±0.02); GST-NefEE155QQ, 0.25 (±0.04); GST-NefEE155QQ, 1.05 (±0.09).
these targets. In ldlF cells at 34°C, the viral protein targeted CD4 to markedly acidic vesicles (mean pH: 5.4) (Figure 7), as previously observed in 293 cells (Figure 5). Furthermore, these vesicles had a predominantly perinuclear localization compatible with that of late endosomes/lysosomes (not illustrated; Gu et al., 1997). In contrast, at the nonpermissive temperature of 40°C, internalized CD4 molecules were found in vesicles with a mean pH of 6.6 (Figure 7) and exhibited mostly the peripheral distribution typical of early endosomes (not shown).

Taken together, these results support a model in which the Nef protein of HIV-1 targets CD4 to lysosomal degradation by acting as a connector between the receptor and the β subunit of COPI coatomers in the endosomal compartment.

Discussion

This work elucidates the mechanism of Nef-induced CD4 degradation and reveals that β-COP, a component of COPI coatomer, is a sorting molecule that can recognize acidic-based lysosomal targeting signals in the cytoplasmic domain of endosomal proteins.

Mechanism of Nef-Induced CD4 Degradation

The observation that some Nef mutants can downregulate the cell surface expression of CD4 without altering its endocytosis led to the discovery that the viral protein independently diverts the receptor to a degradative pathway. Reciprocally, an HIV-1 Nef mutant that induces high levels of CD4 internalization, yet is markedly defective for CD4 downmodulation, allowed the characterization of a Nef sequence specifically involved in lysosomal targeting. This motif is based on an acidic dipeptide (EE) found in most HIV-1, HIV-2, and SIV Nef alleles. The Nef protein of all three viruses can bind the plasma membrane adaptor complexes, and this interaction plays a pivotal role in triggering CD4 endocytosis (Greenberg et al., 1998; Le Gall et al., 1998; Piguet et al., 1998). However, Nef-induced CD4 degradation stems from the recognition of a distinct downstream partner, β-COP. Mutating the EE motif of HIV-1 Nef abrogates its ability both to target CD4 from the early to the late endosomal compartment and to interact with β-COP, yet does not affect AP binding nor Nef-induced CD4 endocytosis. While a wild-type CD4-Nef chimera accumulates mostly in late endosomes and lysosomes following internalization, an EE-mutated derivative localizes preferentially to early endosomes and is rapidly recycled to the cell surface.

At Least Four Nef Determinants Directly Involved in CD4 Downmodulation

Taken together with those of previous studies, these results delineate in HIV-1 Nef four distinct determinants crucial for efficient CD4 downregulation, each carrying on one step of this process (Figure 8). The N-terminal myristoylation signal of Nef allows for its concentration on membranes, the primary site of Nef action. A second domain, centered around amino acids 57-59, binds the CD4 cytoplasmic tail. A third motif, which includes the
Acidic Motifs as Lysosomal Targeting Signals

The acidic dipeptide of Nef thus functions as a lysosomal targeting signal through its recognition by β-COP. Acidic-based motifs have been previously shown to play an important role in the intracellular trafficking of proteins. An acidic cluster in furin and in the mannose-6-phosphate receptor (MPR) mediates TGN retrieval by connecting the cytoplasmic domain of these proteins with PACS-1, a cytosolic sorting molecule (Voorhees et al., 1995; Wan et al., 1998). In addition, acidic residues are often part of the signal that specifies basolateral targeting in epithelial cells (Matter et al., 1994). They can also participate in the dileucine motif-based targeting to the endocytic pathway of the invariant chain of major histocompatibility complex class II molecules (Pond et al., 1995). The present study reveals a yet unsuspected role for acidic motifs in demonstrating that a 15-amino-acid sequence of HIV-1 Nef centered around a critical EE dipeptide mediates transport from early endosomes to late endosomes. This function may be relevant for a variety of cellular proteins, since acidic motifs are widely distributed (Wan et al., 1998).

Uncoupling between Endocytosis and Lysosomal Targeting

Sequences that are necessary for the lysosomal targeting of membrane proteins have been identified by mutagenesis. They most often belong to the family of tyrosine- or dileucine-based endocytosis signals that mediate CCP incorporation (Peters et al., 1990; Letourneur and Klausner, 1992; Marks et al., 1995). In these cases, mutations usually affect both the internalization and the lysosomal degradation of the protein. Exceptions to this rule are P-selectin (Green et al., 1994), the cytoplasmic tail of which contains a degradation signal that encompasses a critical leucine residue, and the IL-2 receptor β chain, in which an α-helical domain mediates lysosomal sorting after endocytosis (Subtil et al., 1997). A dissociation between determinants of internalization and degradation has also been suggested for the epidermal growth factor receptor (EGFR) (Opresko et al., 1995). However, Nef offers the remarkable example of a protein in which endocytosis and lysosomal sorting signals are not only distinct but also well defined, in terms both of their sequence and of their respective downstream target.

Remarkably, the endocytosis and lysosomal targeting functions of HIV-1 Nef map to motifs situated in the same exposed loop of the protein, within a very short distance from one another (Grzesiek et al., 1996a; Lee et al., 1996). This strongly suggests that adaptins and β-COP, the respective ligands of these two signals, bind to Nef sequentially rather than jointly. That is also what is expected from the fact that these two cellular proteins are involved in consecutive rather than simultaneous steps of Nef action. Some regulatory mechanism is probably involved in the differential binding of adaptins and β-COP to Nef. In that respect, it is interesting to note that the recruitment of β-COP to the membrane of endosomes is dependent on the acidification of the luminal pH (Aniento et al., 1996). It might have been that the reported binding of Nef to a subunit of the endosomal proton pump participates in creating local conditions more favorable for β-COP binding. However, the finding that efficient lysosomal targeting of a chimeric integral membrane protein can be conferred by adding a Nef sequence (amino acids 151–165) that excludes residues (amino acids 173–174) reportedly critical for the binding of this cellular protein (Lu et al., 1998) argues against such a possibility.

β-COP as an Endosomal Sorting Molecule

COPI vesicles have long been known to mediate the anterograde and retrograde transport of proteins between the endoplasmic reticulum (ER) and the Golgi. COPI vesicles are formed following the membrane recruitment of a precursor complex, the coatomer. Coatomer consists of seven subunits (α, β, β′, γ, δ, ε, and η) and binds to target membranes in association with ARF, a small GTP-binding protein. Recent studies have revealed that COPI proteins, with the exceptions of the γ and δ subunits, also associate with endosomes, suggesting that coatomer participates in sorting at this level as well. Supporting this proposal, the formation of intermediates (ECV/MVB) that mediate transport from early to late endosomes depends on β-COP and on the endosomal COP complex both in vivo and in vitro (Aniento et al., 1996). Also, the microinjection of β-COP-specific antibodies blocks the entry of vesicular stomatitis virus (VSV), an agent that penetrates cells via the endocytic pathway (Whitney et al., 1995). Finally, formation of ECV/MVB and transport from early to late endosomes are inhibited at the restrictive temperature in the mutant CHO line IdIF with a temperature-sensitive defect in ε-COP in vivo, and ECV/MVB formation is inhibited in vitro by cytosol prepared from IdIF cells incubated at the restrictive temperature (Gu et al., 1997). Interestingly, in these cells the sorting of the EGF receptor from the early to the late endosomes was reduced although no evidence indicated if this effect was a direct result of the defect in the COPI coatomer (Daro et al., 1997). It is therefore relevant that the Nef-induced targeting of CD4 to the degradative pathway is inhibited in IdIF cells at the restrictive temperature. Another report indicates that the cytoplasmic domain of the PDGF can interact with COPI coatomer subunits, although to our knowledge, no function has been attributed to that interaction (Hansen et al., 1997).

The present study not only confirms the participation of β-COP in endosomal transport but also reveals that the coatomer component plays this role as a sorting molecule. In the biosynthetic pathway, dilysine-based (KXXX) or aromatic residue-containing motifs in the cytoplasmic domain of certain proteins are recognized by α-ε- and ε-COP, or by δ-COP, respectively, which results in their ER retrieval (Cosson and Letourneur, 1994; Cosson et al., 1998). We show here that a distinct type of sequence, based on an acidic dipeptide, is recognized by β-COP in endosomes, and as such functions...
as a lysosomal targeting signal. It will be interesting to search for this type of sequence in the cytoplasmic region of proteins that are routed to degradation following endocytosis or in endosomal membrane constituents that might serve as \( \beta \)-COP receptors in this compartment.

The Nef-mediated recruitment of AP complexes triggers the formation of CCPs that preferentially internalize CD4 (Foti et al., 1997). By analogy, it is tempting to postulate that Nef also induces the de novo generation of carrier pH values involved in transporting the receptor from early endosomes to late endosomes and lysosomes. The further study of Nef-induced CD4 lysosomal targeting is likely to reveal important information on the mechanisms by which these vesicles are normally formed and in particular on the modalities by which coatamer is involved in this process critical for the biology of all cells.

**Experimental Procedures**

**DNA Constructions**

Plasmids CMX-Nef1 and CMX-CD4-Nef chimeras, in which expression is driven from the CMV immediate-early promoter, were previously described (Aiken et al., 1994; Mangasarian et al., 1997). Mutations in the HIV-1 Nef coding sequence were generated by PCR-mediated site-directed mutagenesis and then cloned in the pCMX context. pGEX HIV-1 Nef wild-type and mutant constructs were generated by PCR using pGEX-2T (Pharmacia) as the backbone and used to produce GST fusion proteins. The Myc-tagged \( \beta \)-COP eukaryotic expression vector pSGS-Myc-\( \beta \)-COP was a gift from T. Kreis (University of Geneva, Geneva, Switzerland). A portion (amino acids 470-933) was subcloned by PCR in the pGEX-2T plasmid. The 44-TAC chimeras were generated by annealing long complementary oligonucleotides and ligating them in a BamHI-NotI cut pCMX-44Nef expression vector, substituting the Nef fragment to the TAC cytoplasmic domain.

**Antibodies**

The mouse monoclonal anti-\( \beta \)-COP antibody M3AS was a gift of T. Kreis. The antibodies against all other COP subunits were a gift of C. Harter (Heidelberg, Germany). The mouse monoclonal antibody against the \( \alpha \) chain of adaptor complexes was from Sigma (100/2). The conjugated monoclonal antibodies against CD4 were from DAKO.

**Cell Lines and Transfections**

293T cells were provided by G. Nolan (Stanford University) and grown in DME supplemented with 10% fetal calf serum (FCS). CHO (Chinese hamster ovary) and IdIF mutant cells were obtained from M. Krieger (Massachusetts Institute of Technology, Cambridge, MA) and grown in F12 medium with 10% FCS as described (Gu et al., 1997). Transfections were performed using the calcium phosphate method (Alken et al., 1994).

**Endocytosis and Recycling Experiments**

The FACScan-based endocytosis and recycling assays were described in details previously (Mangasarian et al., 1997; Piquet et al., 1998).

**pH Measurements in Endosomes**

Endosomal pH was measured by ratio fluorescence imaging of an internalized pH-sensitive antibody, as described (Demarex et al., 1998). 293T cells grown on glass coverslips were incubated with a FITC-conjugated CD4 antibody for 45 min at 37°C, acid washed three times at 4°C, inserted into a perfusion chamber (Medical Systems Corp., Greenvale, NY), and imaged at 37°C in 1 ml PBS supplemented with 1 mM CaCl\(_2\), 1 mM MgCl\(_2\). Dual excitation ratio imaging was performed on a Zeiss Axiosvert microscope equipped with a cooled CCD camera (VisiTron Systems, Puchheim, Germany), using a 100×/1.3 NeoFluar objective and 2×2 binning to achieve a final resolution of 134 nm per pixel. Images were acquired for 500 ms at \( \lambda = 490 \pm 6 \) nm and for 1 s at \( \lambda = 440 \pm 6 \) nm, using a DeltaRAM monochromator (PTI, Monmouth Junction, NJ), a 510 DRLP dichroic mirror (Omega Optics, Brattleboro, VT), and a 535±25 nm emission filter (Omega). The MetaFluor 3.5 software (Universal Imaging, West Chester, PA) was used to control image acquisition and wavelength selection. Calibration and image processing were performed as previously described (Demarex et al., 1998). Quantification of cell-associated fluorescence was obtained using the Metamorph/Metafluor package (Universal Imaging, West Chester, PA). Data were graphed using the Origin software (MicroCal Software Inc., Northampton, MA) and are shown as means±one standard error of the mean. LDIF transfected with CD4 and Nef were grown on coverslips at 34°C and either placed for 6-12 hr at the restrictive temperature (40°C). They were incubated with a FITC-conjugated CD4 antibody for 45 min at 34°C, washed three times with PBS, chased for 30 min at 34°C, acid washed three times at 4°C, inserted into a perfusion chamber, and analyzed as described above.

**Immunoelectron Microscopy**

Ultrastructural studies were performed as previously described (Piquet et al., 1998). In brief, transiently transfected 293T cells expressing either CD4 or the wild-type or mutant CD4-Nef chimeras were incubated 2 hr at 4°C with a primary mouse anti-CD4 antibody (Leu3a, 40 ng/10\(^6\) cells, Becton Dickinson), followed by anti-mouse IgG coupled to 10 nm colloidal gold particles (Sigma). After two washes in cold PBS, cells were warmed for 10 min at 37°C, fixed 30 min at room temperature with 2.5% glutaraldehyde in phosphate buffer (pH 7.4), dehydrated, and processed for thin section electron microscopy. Gold particles were quantitatively analyzed on cells judged to be morphologically well preserved. For each transfected population, about 1500-3600 gold particles were analyzed from 15-30 cells.

**In Vitro Binding Assays**

Total bacterial extracts expressing HIV-1 Nef (Aiken et al., 1996) and mutants fused to glutathione-S-transferase (GST) were generated and purified on glutathione beads according to the manufacturer's instructions (Pharmacia). \( ^{35} \)-S-methionine-labeled \( \beta \)-COP was prepared by in vitro transcription/translation (Promega). For binding studies, 10 \( \mu \)l of in vitro-transcribed/translated \( \beta \)-COP was mixed with 15 \( \mu \)l of GST-beads, 20 \( \mu \)g of GST-proteins in a total of 1 ml of PBS, for 2 hr at room temperature. The pellet was washed five times for 5 min in PBS, BSA 1%, NaCl 0.2 M, Triton X-100 0.1% (wash buffer) and analyzed by SDS-PAGE and autoradiography. For capture of whole COP coatamers, the GST-fusion proteins were not eluted from the beads, but rather incubated with cytoplasmic extract from CHO cells prepared as described in Gu et al. (1997). Eighty microliters of CHO cell cytosol, containing between 500 and 1000 \( \mu \)g of protein, was used in each binding reaction together with 20 \( \mu \)g of GST proteins in 15 \( \mu \)l of GST beads, in PBS, for 2 hr at room temperature. Following five 5 min washes, bound proteins were eluted and analyzed by SDS-PAGE and Western blotting with the appropriate antibodies. Capture of whole adaptor complexes was performed as previously described (Piquet et al., 1998). Quantification on films was performed using the ONE D-Scan program (Scanners). COP-enriched and -depleted fractions (Aniento et al., 1996) were analyzed in detail previously (Mangasarian et al., 1997; Piguet et al., 1998).

**Acknowledgments**

We would like to thank especially the laboratory of the late T. Kreis, including A. Whitney and M. Gomez, for the gift of plasmids and...
antibodies against β-COP, as well as for useful discussions. We thank M. Marsh, P. Cosson, and A. Mangasarian for insightful comments, and G. Nolan, C. Harter, F. Wieland, M. Krieger, and Y. Chen for providing various reagents. V. P. is the recipient of a M.D.-Ph.D. scholarship from the Swiss National Science Foundation. This work was supported by grants from the Swiss National Science Foundation to D. T., J. G., J.-L. C., and N. D., from the OFSP to D. T. and J. L. C., and by NIH award R37 AI 34306-05 and a professorship from the Gabriella Giorgi-Cavagliari Foundation to D. T.

Received October 28, 1998; revised February 26, 1999.

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