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Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes

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The Nef protein of primate lentiviruses down-regulates the cell surface expression of CD4 and probably MHC I by connecting these receptors with the endocytic machinery. Here, we reveal that Nef interacts with the μ chains of adaptor complexes, key components of clathrin-coated pits. For human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus (SIV) Nef, this interaction occurs via tyrosine-based motifs reminiscent of endocytosis signals. Mutating these motifs prevents the binding of SIV Nef to the μ chain of plasma membrane adaptor complexes, abrogates its ability to induce CD4 internalization, suppresses the accelerated endocytosis of a chimeric integral membrane protein harboring Nef as its cytoplasmic domain and confers a dominant-negative phenotype to the viral protein. Taken together, these data identify μ adaptins as downstream mediators of the down-modulation of CD4, and possibly MHC I, by Nef.

Keywords: adaptor complexes/CD4/lentivirus/MHC class I/Nef

Introduction

The Nef protein of human and simian immunodeficiency viruses (HIV and SIV, respectively) is an important virulence factor which promotes high titer virus replication *in vivo* and precipitates the occurrence of AIDS (Kestler *et al*., 1991; Deacon *et al*., 1995; Kirchhof *et al*., 1995; Collins *et al*., 1998). As a membrane-associated cytoplasmic protein that is produced at the earliest stage of viral gene expression, Nef down-regulates the cell surface expression of major histocompatibility complex class I (MHC I) and of CD4 (Guy *et al*., 1987; Garcia and Miller, 1991; Schwartz *et al*., 1996). While MHC I down-regulation appears to protect HIV-infected cells from efficient recognition and killing by cytotoxic T lymphocytes (Scheppler *et al*., 1989; Collins *et al*., 1998), recent evidence indicates that Nef-induced CD4 modulation contributes to enhancing virion infectivity by preventing the sequestration of the viral envelope by its receptor (J.Lama and D.Trono, submitted).

The Nef-induced down-regulation of CD4 and MHC I reflects a combination of accelerated endocytosis and, at least for CD4, some direct routing from the Golgi apparatus to the endocytic pathway (Aiken *et al*., 1994; Rhee and Marsh, 1994; Schwartz *et al*., 1995, 1996; Mangasarian *et al*., 1997). The endocytosis of membrane-associated receptors normally proceeds through the recognition of specific motifs within their cytoplasmic tail by clathrin-coated pits (CCPs) (Goldstein *et al*., 1985; Rodman *et al*., 1990). The assembly of CCPs at the plasma membrane and on the *trans*-Golgi network (TGN) requires an interaction between cytosolic clathrin and protein complexes called adaptors, or APs (reviewed in Robinson, 1994). Three closely related classes of clathrin-associated APs are found in the cell. The first two (AP-1 and AP-2) mediate protein sorting in the *trans*-Golgi and at the plasma membrane, respectively. The third one (AP-3) was shown recently to participate in cargo-selective transport from the Golgi to the lysosome (Cowles *et al*., 1997). All APs are heterotetramers made of two large subunits of ~100 kDa (γ and β′ for TGN APs, α and β for plasma membrane APs, β3 and δ for AP-3), a medium chain of ~50 kDa (μ1 or AP47 for AP-1, μ2 or AP50 for AP-2 and μ3 for AP-3), and a small chain of ~20 kDa (σ1, σ2 and σ3, respectively).

Adaptor complexes not only mediate the formation of CCPs, but also recruit receptors that carry internalization signals in their cytoplasmic tail. Many of these signals consist of continuous sequences of 4–6 amino acids which contain either a critical tyrosine residue or a dileucine repeat (Letourneur and Klausner, 1992; Trowbridge *et al*., 1993). Prototypic tyrosine-based endocytosis signals, for instance, consist of a YXXØ sequence, where Y is a tyrosine, X is any residue, and Ø is an amino acid with a bulky hydrophobic side chain (Trowbridge *et al*., 1993). Although the identity of the AP component responsible for recognizing each variety of endocytosis signal has not been determined systematically, recent evidence indicates that a number of tyrosine-based motifs specifically interact with the μ1 and μ2 medium chains of adaptor complexes (Ohno *et al*., 1995, 1996; Rapoport *et al*., 1997).

Nef-induced down-modulation also depends on the presence of specific signals within the cytoplasmic domain of its targets. A dileucine-based motif and a critical tyrosine residue are thus essential for the Nef responsiveness of CD4 and MHC I, respectively (Aiken *et al*., 1994; Salghetti *et al*., 1995; S.Le Gall and O.Schwartz, personal communication). This requirement, however, can be circumvented by fusing Nef to the transmembrane and extracellular portions of integral membrane proteins such as CD4 or CD8. The resulting chimeric molecules, in spite of lacking receptor-derived endocytosis signals, undergo accelerated internalization via CCPs (Mangasarian *et al*., 1997). Based

on this evidence, we proposed that Nef down-regulates CD4, and probably MHC I, by acting as a physical connector between these molecules and intracellular trafficking pathways (Mangasarian *et al*., 1997). This model has two implications. First, Nef must interact with CD4 and MHC I, either directly or indirectly. Second, the viral protein must carry signals that are recognized by some component of the endocytic machinery.

Although no data are yet available for the direct binding of Nef to MHC I, an interaction between Nef and CD4 was detected in insect cells overexpressing the two proteins, and using a GST–Nef capture assay on human T cell extracts (Harris and Neil, 1994; Greenway *et al*., 1995). Furthermore, the dileucine-dependent binding of Nef to the membrane-proximal portion of the CD4 cytoplasmic tail was demonstrated in the yeast two-hybrid system as well as *in vitro* by nuclear magnetic resonance (Grzesiek *et al*., 1996; Rossi *et al*., 1996).

Here, we provide evidence that fulfills the second requirement of our model, by revealing that Nef binds to the μ chain of adaptor complexes, and that this interaction is important for CD4 down-regulation. Nef thus acts as a connector molecule that bridges specific receptors with a key component of CCPs.

Results

Interaction between Nef and the μ chain of adaptor complexes

Previous experiments demonstrated that Nef acts as a connector with CCPs (Mangasarian *et al*., 1997) and that the cytoplasmic tail of some receptors is recognized by the μ chain of adaptor complexes (Ohno *et al*., 1995). Based on this dual premise, the possibility that Nef interacts with μ adaptin was investigated, first using the yeast two-hybrid system as described (Ohno *et al*., 1995). Chimeric proteins comprising the Gal4 DNA activation domain and either full-length or truncated (amino acids 121–435) versions of the murine μ2 adaptin (3M2 and 3M9, respectively) or the full-length μ1 adaptin (AP47) were utilized as targets (Ohno *et al*, 1995). Fusion proteins between the Gal4 DNA-binding domain and HIV-1_{R7}, HIV-1_{LAI}, HIV-2_{ROD} or SIV_{MAC239} Nef served as baits. Analogous constructs containing either the prototypic SDYQRL endocytosis motif from the cytoplasmic tail of the integral membrane protein TGN38 or its inactive version SDGQRL served as positive and negative controls, respectively, as described (Ohno *et al*., 1995). Based on the ability of transformed yeast colonies to express a Gal4-dependent β-galactosidase (β-gal) reporter and to grow on histidine-deficient plates, a strong and specific interaction was detected between the full-length 3M2 or the truncated 3M9 μ2 adaptin derivatives and HIV-2 or SIV Nef. Interactions with AP47 were weaker, in particular for HIV-2 Nef. Yeast clones expressing the adaptor medium chains and HIV-1 Nef did not grow on His-deficient medium, and expressed only low levels of β-gal activity (Table I). In addition, no binding was observed between HIV-1 Nef and the α, β2, γ, σ1 and σ2 chains of adaptor complexes (data not shown).

The N-terminal domains of the HIV-2_{ROD} and SIV_{MAC239} Nef proteins contain one and two stretches of amino acids, respectively, that are reminiscent of the canonical YXXØ endocytosis signals: Y^{39}SRF in HIV-2_{ROD} and Y^{28}GRL/Y^{39}SQS in SIV_{MAC239}. Interestingly, no such motif is found in the corresponding region of HIV-1 Nef (Figure 1). Replacing tyrosine by glycine at positions 39 of HIV-2 Nef or 28 and 39 of SIV abrogated the interaction of Nef with μ2 adaptins in the yeast two-hybrid system, as did partly the substitution of alanine for Leu31 and Leu32 of SIV Nef (Table I). These two residues could form a dileucine-based signal, but Leu31 is also part of the proximal putative tyrosine-based motif of SIV Nef ($YGRL_{31}$). Interestingly, replacing this residue by alanine severely decreased the binding of Nef to μ2, whereas mutating Leu32 only had a modest effect. All of the amino acid substitutions tested also impaired the interaction between Nef and AP47, in particular the double tyrosine substitution in SIV Nef, although some residual binding was noted (Table I).

To corroborate the results obtained in the yeast two-hybrid system, the ability of *in vitro* translated 3M9 to interact with HIV-1 and SIV Nef proteins fused to GST was evaluated (Figure 2A–C). 3M9 was used instead of full-length μ2 because this truncated form was shown previously to have a higher affinity for tyrosine-based motifs in binding studies (Ohno *et al*., 1996). A strong interaction between SIV Nef and the μ2 derivative could be demonstrated (Figure 2A). Furthermore, a fusion protein in which only 59 N-terminal amino acids of SIV Nef were appended to GST (GST–SIV $Nef_{1–59}$) could capture 3M9 (Figure 2B). Mutating Tyr28 and Tyr39 of SIV Nef abolished the SIV Nef–μ2 interaction, within the context of both full-length Nef and of N-terminal fragment (Figure 2A and B). In contrast, replacing Asp204 by lysine, a change previously demonstrated to abrogate Nef-induced CD4 down-regulation (Iafrate *et al*., 1997), had no effect (Figure 2A). Finally, a specific interaction between *in vitro* translated 3M9 and GST–HIV-1 Nef fusion proteins could also be demonstrated, but this interaction appeared to be weaker and was best detected under low salt conditions (Figure 2C).

Lastly, the ability of the GST–Nef fusion proteins to recruit whole adaptor complexes was tested as previously described (Dietrich *et al*., 1997). GST–SIV $Nef_{1–59}$ could capture α and β adaptins present in cytoplasmic extracts of human Jurkat T lymphoid cells (Figure 2D and data not shown). This interaction was prevented either by mutation of Tyr28 and Tyr39 in Nef or by addition of peptide corresponding to a wild-type but not to a mutated μ-binding endocytosis signal (Figure 2D). Similarly, a GST–HIV-1 Nef fusion protein could recruit the α and β chains of adaptor complexes, and this binding could also be competed with specific peptide, suggesting that it too occurred through μ adaptin (Figure 2E).

Efficient CD4 down-regulation requires Nef binding to μ2 adaptin

The importance of the Nef–μ2 interaction for CD4 down-regulation was assessed by transient transfection as previously described (Aiken *et al*., 1994, 1996). Wild-type or mutated versions of SIV Nef were produced in 293T human kidney cells together with CD4, and cell surface levels of the glycoprotein were determined by fluorescence-activated cell sorting (FACS). All SIV Nef variants were expressed comparably as assessed by Western blot

Table I. Nef–μ interactions in the yeast two-hybrid system

		pGBT9 Clones															
		Controls				HIV-1 Nef		HIV-2_{ROD} Nef		SIV_{MAC239} Nef							
		$(SDYQRL)_3$	$(SDGQRL)_3$	Empty	HIV-1MA	WT (R7)	WT (LAI)	WT	$Y^{39}G$	WT	$Y^{28}G$	$Y^{39}G$	$L^{31}A$	$L^{32}A$	$LL^{32}AA$	$YY^{28/39}GG$	$D^{204}K$
pACTII Clones	3M2	++++ / +++	– / –	– / –	– / –	– / –	+ / –	+++ / +++	– / –	+++ / ++	+/– / –	++ / ++	+/– / –	++ / ++	+/– / –	– / –	++ / ++
	3M9	++++ / +++	– / –	– / –	– / –	– / –	+ / –	+++ / +++	– / –	+++ / ++	– / –	++ / ++	– / –	++ / +	– / –	– / –	++ / +
	AP47	++++ / +++	– / –	– / –	– / –	+/– / –	+/– / –	+ / –	+/– / –	+++ / ++	++ / +	++ / +	++ / +	++ / +	+/– / –	+/– / –	++ / +
	NIPD			– / –	– / –	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++	++++ / +++

Yeast Y190 cells were co-transformed with the indicated plasmids, and evaluated for their ability either to express a Gal4-dependent β-gal reporter or to grow on His-deficient medium. pACTII clones encode fusion proteins comprising the Gal4 activation domain, pGBT9 clones encode fusion proteins containing the Gal4 DNA-binding domain. 3M2 and 3M9 represent full-length and N-terminally truncated versions of the plasma membrane μ adaptin (μ2), AP-47 the μ chain of the TGN adaptor complexes (μ1). NIPD is a previously cloned Nef-interacting protein. In each case, scores given in the upper left portion of the box represent levels of β-gal expression, and scores given in the lower right portion growth on His^- medium. For the β-gal assay, the intensity of the interaction is reflected by the speed at which the blue color develops and was scored as follows: ++++, color developed within 1 h of incubation at 30°C; +++, color developed within 4 h of incubation; ++, color developed after overnight incubation; +, color developed after 2–3 days of incubation; +/–, faint color developed after 2–3 days of incubation; and –, no color developed after 3 days of incubation. For the growth on His^- medium: +++, growth observed within 1–3 days of plating; ++, growth observed within 4–5 days of plating; +, growth observed after 6–8 days of incubation; –, no appreciable growth detected after 8 days of incubation.

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1   MGGKWSKSSVIGWPTVRERMRRAEPAA   HIV-1 (R7)
1   MGGKWSKSSVVGWPTVRERMRRAEPAA   HIV-1 (LAI)
1   MGASGSKKHSRPPRGLQERLLRARAGA   HIV-2 (ROD)
1   MGGAISMRRSRPSGDLRQRLLRARGET   SIV (MAC239)

28  ------------DGVGAVSRDLE---   HIV-1 (R7)
28  ------------DGVGAASRDLE---   HIV-1 (LAI)
28  CGGYWNESGGEYSRFQEGSDREQKSPS   HIV-2 (ROD)
28  YGRLLGEVEDGYSQSPGGLDKGLSSLS   SIV (MAC239)
    *  **       *

39  ----------------KHGAITSSNTA   HIV-1 (R7)
39  ----------------KHGAITSSNTA   HIV-1 (LAI)
55  CEGRQYQQGDFMNTPWKDPAAEREKNL   HIV-2 (ROD)
55  CEGQKYNQGQYMNTPWRNPAEEREKLA   SIV (MAC239)

50  ---ATNADCAWLEAQEEEEVGFPVTPQ   HIV-1 (R7)
50  ---ATNAACAWLEAQEEEEVGFPVTPQ   HIV-1 (LAI)
82  YRQQNMDD---VDSDDDDQVRVSVTPK   HIV-2 (ROD)
82  YRKQNMDD---IDEZDDDLVGVSVTPK   SIV (MAC239)
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Fig. 1. Amino acid sequence alignment of the N-terminal regions of $HIV\text{-}1_{R7}$ Nef, $HIV\text{-}1_{LAI}$ Nef, $HIV\text{-}2_{ROD}$ Nef and SIV_{MAC239} Nef (in single-letter code). Tyrosine-based motifs in HIV-2 and SIV Nef are in bold characters; asterisks underline HIV-2 and SIV Nef residues mutated in various experiments.

analysis (Figure 3A). Wild-type SIV Nef decreased the cell surface levels of CD4 ~10-fold, while it did not affect a dileucine-mutated CD4 variant (not illustrated). Replacing Tyr28 or Tyr39 of Nef by glycine decreased this effect by 40 and 20%, respectively, while mutating both residues inhibited Nef function by 75% (Figure 3B). The substitution of alanine for leucine at positions 31 and 32 also decreased SIV Nef-induced CD4 down-regulation, consistent with the participation of these residues in adaptin binding, but it did not have any additional effect over that obtained with the double tyrosine mutation. Finally, replacing Asp204 by lysine (in a mutant called $D^{204}K$) completely abrogated the effect of SIV Nef on CD4, as previously reported (Iafrate *et al.*, 1997).

To confirm these results, a FACS-based CD4 endocytosis assay was utilized as previously described (Mangasarian *et al.*, 1997) (Figure 4). The Nef effect was most pronounced during the early times of the analysis, where wild-type SIV Nef increased the fraction of CD4 molecules internalized by ~3-fold (between 0 and 2.5 min, 6.3% per min in Nef-producing cells versus 2% per min in control cells). At 20 min, 36% of the CD4 molecules originally present on the surface of Nef-expressing cells were found inside the cells, compared with 18% in cells transfected with a control vector. Both the $YY^{28/39}GG$ and $D^{204}K$ mutations abrogated the ability of Nef to accelerate CD4 endocytosis *in trans*, consistent with a model in which the residues affected by these changes are involved in connecting Nef with the endocytic machinery and with CD4, respectively.

Distinction between μ2-binding and CD4-recruiting domains of Nef through the use of Nef-containing chimeric integral membrane proteins

To investigate this model further, chimeric integral membrane proteins which comprised the extracellular and transmembrane regions of CD8 with SIV Nef as their cytoplasmic domain were expressed in 293T cells by transient transfection. As previously observed with comparable HIV-1 Nef-derived CD4 or CD8 chimeras (Mangasarian *et al.*, 1997), the CD8–SIV Nef fusion protein (called 88SIV Nef) exhibited low cell surface levels compared with a tail-less CD8 variant (88X) even though both molecules were highly expressed inside the cells (Figure 5). In this context, the $D^{204}K$ mutation had no effect, consistent with the predicted involvement of this residue in CD4 recruitment, rather than in an interaction with the endocytic machinery. In contrast, as expected from the role of the tyrosine-based motifs in μ adaptin binding, the $YY^{28/39}GG$ substitution increased the cell surface expression of 88SIV Nef considerably, albeit to levels that remained lower than those of 88X. 88SIV Nef mutants containing changes at single tyrosine residues or at Leu31 and Leu32 exhibited an intermediate phenotype (not illustrated).

Corroborating these data, both the wild-type and the $D^{204}K$ mutant 88SIV Nef chimeras underwent accelerated endocytosis, while the 88SIV $Nef_{YY}{}^{28/39}{}_{GG}$ variant was internalized with an efficiency only slightly higher than that of a control CD8-based fusion protein which contained the 132 amino acid HIV-1 matrix as its cytoplasmic tail

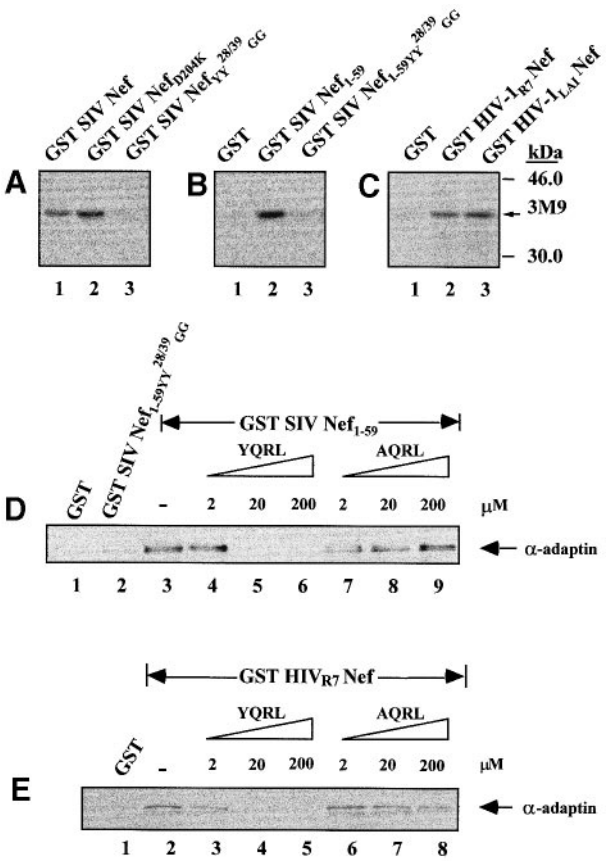

Fig. 2. Interaction between GST–Nef and *in vitro* translated μ2 and whole adaptor complexes. (**A**–**C**) Binding of ^{35}S-labeled *in vitro* translated 3M9 with the indicated GST–Nef fusion proteins. Reactions were perfomred as indicated in Materials and methods. For (A) and (B) (GST–SIV Nef fusions), buffer was as follows: 0.05% Triton X-100, 0.5 mM $MgCl_2$, 20 mM Tris pH 8.0 and 150 mM NaCl. For (C) (GST–HIV-1 Nef fusions), binding buffer was 0.05% Triton X-100, 0.5 mM $MgCl_2$, 20 mM Tris pH 7.5 and 20 mM NaCl. (**D** and **E**) Capture of whole adaptor complexes by GST–Nef. Cytoplasmic extracts from human T lymphoid Jurkat cells were incubated with the indicated GST fusion proteins, with or without addition of wild-type or mutant endocytosis signal-like peptide. Bound material was analyzed by Western blotting with antibodies against α or β adaptins. Only α adaptin is shown, as identical results were obtained with both antibodies.

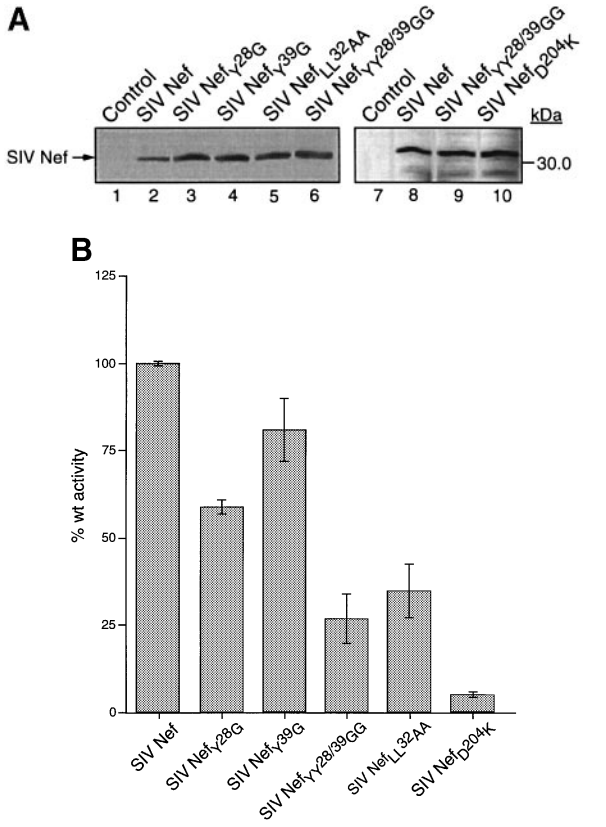

Fig. 3. Adaptin binding is necessary for SIV Nef-induced CD4 down-regulation. (**A**) Western blot analysis of cytoplasmic extracts of 293T cells expressing the indicated SIV Nef variants. (**B**) Relative activity of various SIV Nef derivatives on CD4 down-regulation. Assays were performed by transient transfection in 293T cells followed by FACS analysis of CD4 cell surface levels. Values are the mean of at least four independent assays and are expressed as a percentage of wild-type SIV_{MAC239} Nef activity. Error bars represent 1 standard deviation from the mean.

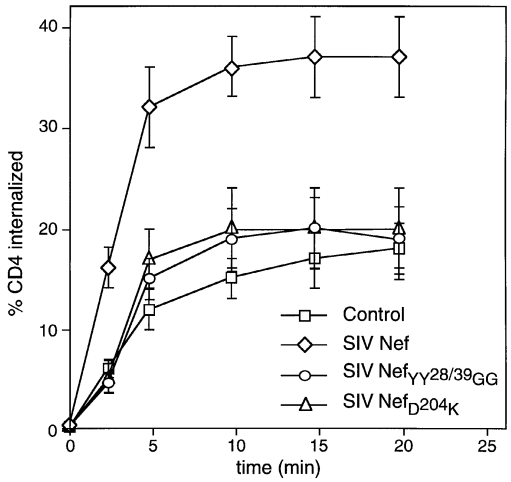

Fig. 4. Induction of CD4 endocytosis by SIV Nef and variants. Internalization rates of the CD4 molecule in the absence and presence of SIV_{MAC239} Nef wild-type or mutated at Tyr28 and Tyr39 or at Asp204 were measured using a FACS-based endocytosis assay. Results are representative of four independent experiments. Error bars represent 1 standard deviation from the mean.

(Figure 6A). This control was chosen to exclude the possibility that the size of the 88Nef cytoplasmic tail was responsible for the increased rates of internalization of this molecule, as previously discussed (Mangasarian *et al*., 1997). The tyrosine-dependent endocytosis of a CD8-based chimera containing HIV-2 Nef as its intracellular domain was also observed, but in this case the Nef effect was partly masked by a lack of stability of the cytoplasmic region of this protein (data not shown).

The results of the *in vitro* binding assays (Figure 2) indicated that 59 N-terminal residues of SIV Nef are sufficient to govern a tyrosine-dependent interaction with μ adaptin. Correspondingly, a CD8-derived chimera comprising only the proximal portion of SIV Nef underwent rates of endocytosis that were equal to or greater than those exhibited by full-length 88SIV Nef. Furthermore, this phenomenon was abrogated by mutating Tyr28 and Tyr39 within the context of this truncated molecule (Figure 6B).

Correspondingly, ultrastructural analyses of transiently transfected 293T cells revealed that the wild-type 88SIV-$Nef_{1–59}$ fusion protein was incorporated in CCPs much

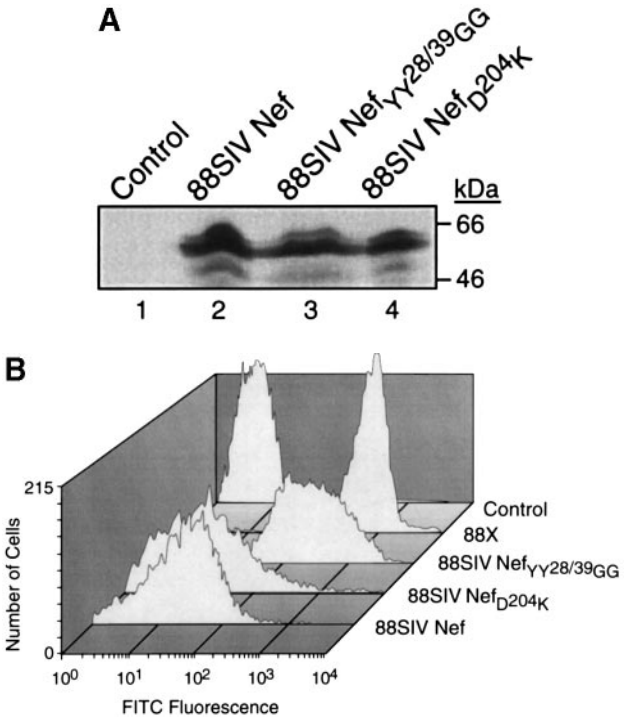

Fig. 5. Cell surface expression of CD8–SIV Nef chimeras. (**A**) Surface levels of CD8 antigenic determinants were evaluated by flow cytometry and are represented by the shaded areas. The control corresponds to cells transfected with an empty vector. The SIV Nef adaptin-binding mutant ($YY^{28/39}GG$) has higher surface levels in the context of a CD8–SIV Nef chimera (88SIV Nef) than the wild-type or the CD4-recruiting $D^{204}K$ mutant. (**B**) Total levels of the CD8–SIV Nef chimeras were determined by Western blotting of cytoplasmic extracts with an SIV Nef-specific antibody.

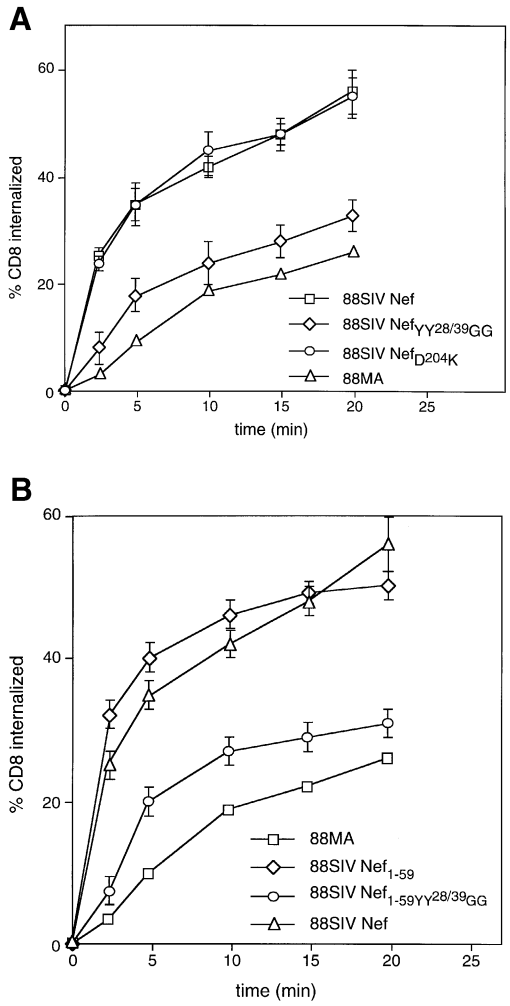

Fig. 6. Endocytosis of CD8–SIV Nef chimeras. (**A**) Internalization rates of the various CD8 chimeras were measured using a FACS-based endocytosis assay. The results are representative of three independent experiments. Error bars are representative of 1 standard deviation from the mean. The SIV $Nef_{YY}{}^{28/39}{}_{GG}$ adaptin-binding mutant shows a reduced internalization in the context of the CD8–Nef fusion protein, while the CD4-recruiting $D^{204}K$ mutant behaves like wild-type. (**B**) Same assay as in (A), with wild-type and tyrosine-mutated forms of a truncated CD8–SIV Nef chimera. The 59 N-terminal residues of SIV Nef are sufficient to induce the tyrosine-dependent accelerated endocytosis of a chimeric integral membrane protein.

more efficiently than its double tyrosine-mutated counterpart (Figure 7). The difference was already visible when the cell surface distribution of these molecules was examined at 4°C immediately after labeling, and it increased further when internalization was allowed to begin by incubating the cells for 5 min at 37°C. This confirmed that the double tyrosine-based motif of SIV Nef is important for interaction with the endocytic apparatus.

SIV Nef adaptin-binding mutant is dominant negative for CD4 internalization

One possible consequence of Nef acting as a connector between CD4 and μ adaptin is that a Nef mutant unable to recognize adaptor complexes might interfere with wild-type Nef function by competing for CD4 access. This hypothesis was tested by measuring the rates of CD4 endocytosis in 293T cells expressing wild-type SIV Nef alone or together with either the $YY^{28/39}GG$ or the $D^{204}K$ mutants. As predicted, the adaptin binding-defective SIV $Nef_{YY}{}^{28/39}{}_{GG}$ variant exhibited a dominant-negative phenotype, whereas $Nef_{D}{}^{204}{}_{K}$, which cannot recruit CD4, did not interfere with wild-type function (Figure 8).

μ2 binding-independent inhibition of CD4 recycling by Nef

The persistence of a significant degree of CD4 down-regulation by the double tyrosine mutant of SIV Nef (Figure 3B), despite its inability to bind μ2 and trigger the accelerated endocytosis of CD4 (Table I; Figures 4 and 6), suggested that Nef might affect the viral receptor through at least one additional, tyrosine-independent mechanism. To investigate this issue, the influence of wild-type and mutant SIV Nef on the cell surface half-life of CD4 was evaluated as previously described (Mangasarian *et al.*, 1997). In 293T cells expressing CD4 alone, the receptor had a cell surface half-life of 10 h, while in the presence of wild-type SIV Nef it decreased to 2 h. In spite of its inability to augment CD4 internalization, the SIV $Nef_{YY}{}^{28/39}{}_{GG}$ variant also decreased the cell surface half-life of the receptor, to 5 h. A possible effect of Nef on the rates of CD4 recycling from the endosome to the plasma membrane was therefore assessed in transiently transfected 293T cells, using a modification of a previously established protocol (Pelchen-Matthews *et al.*, 1989) (Figure 9). In control cells, CD4 was recycled efficiently, with 50% of internalized molecules reappearing on the cell surface

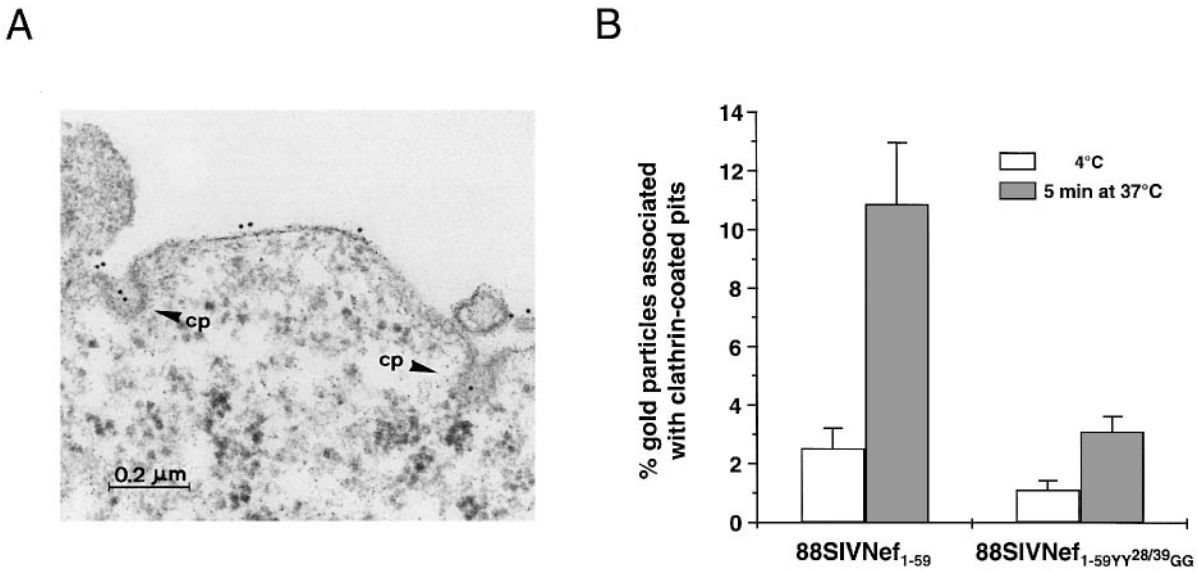

Fig. 7. Tyrosine-dependent CCP recruitment of a Nef-derived chimera. (**A**) Representative electron micrograph showing the association of $88SIVNef_{1–59}$ (gold particles) with CCPs (cp) in transiently transfected 293T cells. (**B**) Percentage of the total number of surface-associated gold particles that were present in CCPs in 293T cells transiently expressing the $88SIVNef_{1–59}$ and $88SIVNef_{1–59YY}{}^{28/39}{}_{GG}$ chimeras. Truncated 88SIVNef chimeras were labeled at 4°C with an anti-CD8 primary antibody followed by a secondary antibody coupled to colloidal gold. Endocytosis of the gold-conjugated complex was then allowed to occur by raising the temperature to 37°C for 5 min. Following cell processing for electron microscopic gold detection, association of gold particles with CCPs was quantified on cells considered well preserved. Gold particles were considered associated with CCPs when they were observed immediately adjacent (at a distance <20 nm) to the clathrin coat or totally enclosed in CCPs.

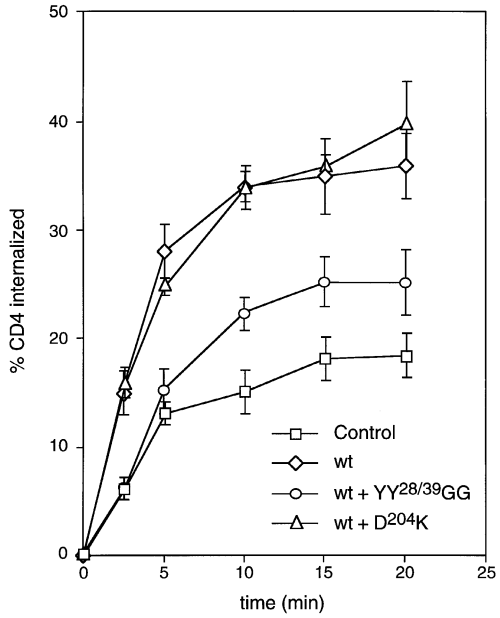

Fig. 8. SIV Nef adaptin-binding mutant is dominant negative for CD4 internalization. Internalization rates of CD4 in the presence or absence of wild-type SIV Nef, with or without addition of the adaptin-binding $YY^{28/39}GG$ or of the CD4-recruiting $D^{204}K$ mutants (at a ratio wild-type to mutant of 1:1), were measured using the FACS-based endocytosis assay. Results are representative of four independent experiments. Error bars are representative of 1 standard deviation from the mean.

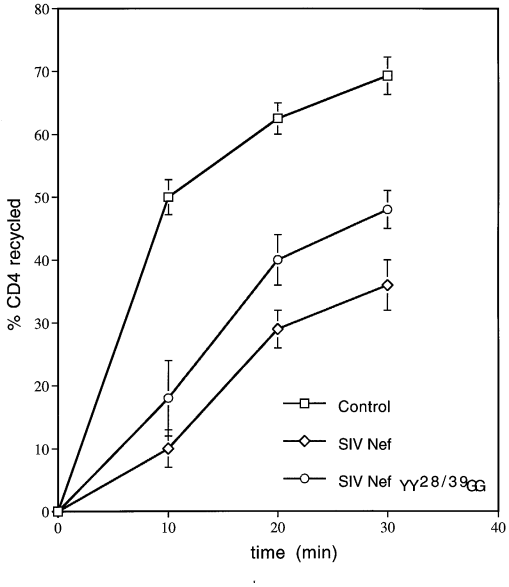

Fig. 9. SIV Nef wild-type and adaptin-binding mutant interfere with CD4 recycling. 293T cells were labeled with phycoerythrin-conjugated monoclonal antibody specific for CD4, and recycling assays were performed as described in Materials and methods. Results are representative of four independent experiments. Error bars correspond to 1 standard deviation from the mean.

within 10 min, consistent with previous measurements of this parameter (Pelchen-Matthews *et al*., 1989). In the presence of wild-type SIV Nef, this fraction was reduced to 10%, while with the SIV $Nef_{YY}{}^{28/39}{}_{GG}$ mutant it was 17%. At later time points, the percentage of recycled CD4 protein increased, but both the wild-type and the tyrosine-mutated variants of SIV Nef still exerted a profound inhibition on this process. In similar experiments, HIV-1 Nef was also found to prevent CD4 recycling. In contrast, and most importantly, Nef did not affect the recycling of transferrin receptor (data not shown).

This last set of results allows one to conclude that Nef down-regulates CD4 both by triggering the accelerated endocytosis of this receptor and by blocking its recycling. Furthermore, our results indicate that only

the first one of these two effects requires the conservation of tyrosine-based sequences important for the binding of SIV Nef to μ adaptin. Interestingly, the truncated CD8–SIV $Nef_{1–59}$ chimera achieved steady-state levels of cell surface expression that were approximately eight times higher than those of its full-length counterpart (data not shown), in spite of exhibiting rates of endocytosis that were comparable (Figure 6). Explaining this discrepancy, the recycling of the full-length CD8–SIV Nef chimera was very inefficient, compared with that of its truncated CD8–SIV $Nef_{1–59}$ version (data not shown). The signal responsible for the lysosomal targeting activity of SIV Nef must therefore include residues that are located downstream of amino acid 59.

Discussion

This work provides strong evidence for a model in which the Nef protein of primate lentiviruses down-regulates the cell surface expression of CD4 by acting as a connector between this receptor and a key component of CCPs, the μ chain of adaptor complexes.

Based on the observation that chimeric integral membrane proteins harboring Nef as their cytoplasmic domain are actively recruited into CCPs in spite of lacking receptor-derived endocytosis signals, we previously suggested that Nef interacts with a component of the cell internalization machinery (Mangasarian *et al*., 1997). Here, the combined results of binding studies and functional analyses identified μ adaptin as the immediate downstream partner of Nef.

An interaction between Nef and μ was first detected in the yeast two-hybrid system. It was confirmed *in vitro* with recombinant molecules and through the capture of adaptor complexes by GST–Nef fusion proteins. The biological relevance of this phenomenon was ascertained by demonstrating that an SIV Nef mutant defective for μ2 binding lost the ability to down-regulate CD4 efficiently. Furthermore, in contrast to wild-type Nef, this mutant failed to trigger the accelerated endocytosis of a chimeric integral membrane protein of which it constituted the cytoplasmic domain. Finally, it behaved as a dominant-negative mutant for CD4 endocytosis.

In the yeast two-hybrid system, the adaptor complex medium chains interacted strongly with HIV-2 and SIV Nef, but only weakly with HIV-1 Nef. This was surprising since HIV-1 Nef down-regulates CD4 as efficiently as its HIV-2 and SIV counterparts (data not shown). For HIV-2 and SIV Nef, μ2 binding was dependent on the presence, in the membrane-proximal portion of these proteins, of tyrosine-based motifs reminiscent of prototypic endocytosis signals. This correlates the recognition by μ2 of similar motifs in the cytoplasmic tails of TGN38, LAMP-1, CD68, H2-Mb and CTL4-A (Ohno *et al*., 1995; Shiratori *et al*., 1997). A comparable sequence is not found in HIV-1 Nef, perhaps explaining the failure to detect a strong interaction between this species of Nef and the adaptor medium chain. Nevertheless, a GST–HIV-1 Nef fusion protein could capture *in vitro* translated μ2, as well as adaptor complexes from the cytoplasm of human lymphoid T cells. Furthermore, the latter interaction could be competed by peptide corresponding to a prototypic μ-binding endocytosis signal. Taken together, these data strongly suggest that μ2 is also a downstream mediator of HIV-1 Nef action. Under physiological conditions, it is possible that post-translational modifications not recapitulated in the yeast two-hybrid system strengthen the HIV-1 Nef–μ2 interaction. Also, the affinity of HIV-1 Nef for the medium chains of adaptor complexes may be increased significantly within the context of clathrin coats, as suggested by the recent demonstration of a cooperativity between receptor–AP-2 interactions and coat formation (Rapoport *et al*., 1997). Future experiments aimed at mapping the region of HIV-1 Nef that is recognized by the endocytic machinery should help to confirm this hypothesis, by allowing the type of functional studies utilized in the present study to validate the significance of the SIV Nef–μ2 association.

The finding that Nef associates with the medium chains of both the plasma membrane and TGN adaptor complexes is consistent with its demonstrated ability to target CD4 to the endosomal compartment from both of these sites (Mangasarian *et al*., 1997). In that respect, it is interesting to note that the double tyrosine mutation which abrogated the interaction between SIV Nef and μ2 adaptin also affected μ1 recruitment (Table I). Nevertheless, whereas Nef efficiently governs the routing of a chimeric integral membrane protein from the Golgi to the endosome, bypassing the cell surface, the transregulation of CD4 by Nef mainly results from accelerated endocytosis from the plasma membrane (Mangasarian *et al*., 1997, and data not shown). The binding of μ1, in that case, may be of minor functional significance.

An SIV Nef mutant unable to bind μ2 was ineffective at inducing the accelerated endocytosis of CD4 (Figure 4), yet it conserved some ability to down-regulate the steady-state cell surface levels of this receptor (Figure 3). Investigating this paradox revealed that this residual activity represented the Nef-mediated inhibition of CD4 recycling. Although it had never been demonstrated formally, this phenomenon corroborates the lysosomal targeting of CD4 that is observed in the presence of Nef (Aiken *et al*., 1994; Rhee and Marsh, 1994).

In contrast to mutations in the μ-binding domain of SIV Nef, the $D^{204}K$ change completely abrogated Nef-induced CD4 down-regulation (Figure 3). This was to be expected from a mutation predicted to block the recruitment of CD4 by the viral protein. The failure of this variant to behave as a dominant-negative mutant for CD4 down-regulation similarly fits with this model.

It is likely that Nef mimics endogenous molecules that are responsible for linking certain cell surface proteins to components of the endocytic machinery. Several cellular proteins have been shown recently to function as connectors between cell surface receptors and CCPs. For instance, β-arrestin appears to act as a clathrin adaptor that facilitates the endocytosis of the β-2 adrenergic receptor (Ferguson *et al*., 1996; Goodman *et al*., 1996), whereas the Eps15 and Shc proteins are thought to participate in bridging the epidermal growth factor (EGF) receptor tyrosine kinase with CCPs (Okabayashi *et al*., 1996; van Delft *et al*., 1997). While β-arrestin binds directly to clathrin (Goodman *et al*., 1996) and Eps15 to α adaptin (Benmerah *et al*., 1996), it is unknown whether other proteins act via μ adaptin. Furthermore, Nef might be unique in that it can induce the *de novo* generation of CCPs, which are at least in part receptor specific (Foti

et al., 1997). CCP formation normally requires not only that adaptor complexes be recruited at the membrane, but also that they become activated to bind clathrin triskelions and initiate the assembly of clathrin lattices (Keen *et al*., 1991; Gallusser and Kirchhausen, 1993; Peeler *et al*., 1993). Future experiments investigating whether the binding of Nef to μ adaptin triggers the activation of adaptor complexes may help to identify the biochemical bases of this process.

Materials and methods

DNA constructions

Plasmids CMX-Nef1 and CMX-SIV_{MAC239} Nef, in which Nef is expressed from the cytomegalovirus (CMV) immediate early promoter, were described previously (Aiken *et al*., 1994; Aiken and Trono, 1995). Vectors producing the CD8-SIV_{MAC239} Nef 88SIVNef and the CD8-HIV-1 MA 88MA chimeras were generated by substituting the SIV *nef* and the HIV-1 MA reading frames, respectively, with the corresponding fragment of HIV-1 *nef* in CMX-88Nef (Mangasarian *et al*., 1997). Constructs pACTII and pGBT9 used for the yeast two-hybrid studies express the Gal4 activation and DNA-binding domain, respectively (Durfee *et al*., 1993; Harper *et al*., 1993; Bartel and Fields, 1995). Plasmids pACTII 3M2, pACTII 3M9, pACTII AP47, pGBT9 $(SDYQRL)_3$ and pGBT9 $(SDGQRL)_3$ were a gift from J.S.Bonifacino (Ohno *et al*., 1995). 3M9 encodes amino acids 121–435 of the murine μ2 adaptin, while 3M2 represents the full-length μ2 (Ohno *et al*., 1995). pGBT9 HIV-1_{R7}Nef, pGBT9 HIV-1_{LAI}Nef, pGBT9 HIV-1_{R7}MA, pGBT9 HIV-2_{ROD}Nef and pGBT9 SIV_{MAC239}Nef were generated by insertion of PCR-generated sequences into pGBT9. HIV-2_{ROD} Nef and SIV_{MAC239} Nef variants were generated by PCR-mediated site-directed mutagenesis of the wild-type sequences and inserted in the various expression vectors. pGEX HIV-1_{R7}Nef, pGEX HIV-1_{LAI}Nef, pGEX SIV_{MAC239}Nef wild-type and mutant constructs were generated by PCR using pGEX-2T (Pharmacia) as the backbone.

Cell lines and transfections

293T cells were provided by G.Nolan (Stanford University) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were performed using the calcium phosphate method (Ausubel *et al*., 1987).

Protein analyses

Western blot analyses and immunoprecipitations were performed as described (Aiken *et al*., 1994). The SIV_{MAC251} Nef monoclonal antibody was obtained from K.Krohn and V.Ovod through the NIH AIDS Research and Reference Reagent Program. The CD8-specific monoclonal antibody was from Pharmingen. α- and β-adaptin-specific antibodies were purchased from Sigma.

Yeast two-hybrid studies

Y190 (Harper *et al*., 1993) yeast cell transformation was performed as described (Ohno *et al*., 1995). pACTII NIPD, used as a positive control, encodes a Nef-interacting protein previously identified by screening a Jurkat cell cDNA library (unpublished). For solid β-gal assays, yeast cells were transformed and incubated for 5 days before transfer onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was dipped into liquid nitrogen to lyse the cells, air dried and carefully laid with the yeast-side up on top of a filter paper wetted with Z-buffer (100 mM Na_2HPO_4, buffered at pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 38 mM 2-mercaptoethanol) supplemented with 1 mg/ml X-Gal. The filter was then incubated at 30°C in the dark and checked periodically for color development. For growth in histidine-deficient medium, single colonies were picked and streaked onto a plate lacking leucine, tryptophan and histidine and supplemented with 10 mM 3-amino-1,2,4-triazole, and the plates were checked periodically for colony growth.

In vitro binding assays

Total bacterial extracts expressing HIV-1_{R7}, HIV-1_{LAI}, SIV_{MAC239} Nef and mutants thereof fused to GST were generated and purified on glutathione beads according to the manufacturer's instructions (Pharmacia). The proteins were eluted out, dialyzed against phosphate-buffered saline (PBS) overnight, stored at 4°C and used within a week for binding studies. [^{35}S]Methionine-labeled 3M9 adaptin chain was prepared by *in vitro* transcription/translation (Promega). For binding studies, 10 μl of *in vitro* transcribed/translated 3M9 were used in a binding reaction containing 15 μl of GST beads, 2 μg of GST proteins in a total of 300 μl of an appropriate binding buffer supplemented with 20 μg of bovine serum albumin (BSA). The binding was carried out at room temperature using a rotating wheel for 1 h. The resulting solution was spun down, and the pellet washed three times for 5 min with 500 μl of the same binding buffer. After binding, proteins were separated by SDS–PAGE for further analysis. For visualization of bound protein, gels were fixed, dried and revealed using autoradiography. For capture of whole adaptor complexes, the GST fusion proteins were not eluted from the beads but instead incubated with cytoplasmic extract from Jurkat T cell lines prepared as described (Dietrich *et al*., 1997). Eighty μl of Jurkat cell cytosol, containing between 500 and 1000 μg of protein, were used in each binding reaction together with 60 μg of GST proteins in 15 μl of GST beads. Binding was carried out in PBS for 2 h at 37°C and washing was done at room temperature three times for 5 min in PBS. Bound proteins were eluted by boiling in SDS–PAGE sample buffer. Eluted material was analyzed by SDS–PAGE and Western blotting with either an anti-α or an anti-β adaptin antibody. Peptides used in the competition assay were chemically synthesized (Applied Biosystems). The wild-type YQRL peptide (sequence KVTRRPKASDYQRLNL) corresponds to the cytoplasmic domain of TGN38 which was shown previously to bind to μ adaptin (Rapoport *et al*., 1997). The mutant AQRL peptide, with the tyrosine replaced by an alanine residue (sequence KVTRRPKASDAQRLNL), is defective for μ adaptin binding.

Flow-cytometric analyses

293T cells were removed from dishes 2 days after transfection by washing once with calcium- and magnesium-free PBS and incubating with 2 mM EDTA in PBS, followed by a wash with DMEM containing 2% FCS. Cells were incubated with the appropriate combination of saturating amounts of phycoerythrin- or fluorescein-conjugated antibodies specific for CD4 or CD8 (DAKO) for 1 h at 4°C. Cells were then washed twice with DMEM containing 2% FCS, and resuspended in 500 μl of PBS. Flow cytometry was performed on a Becton Dickinson FACScan. Live–dead cell discrimination (usually 10–20% of total events) was accomplished by staining the cells with propidium iodide. Data analyses were performed on a Sun workstation using the software package SunDisplay 3 (Joe Trotter). Measurements of CD4 down-modulation were performed as previously reported (Aiken *et al*., 1994, 1996). Co-transfection of a vector expressing the Nef-insensitive CD8 molecule was used to mark transfected cells. The activity of each mutant Nef construct was determined as a percentage of the fold CD4 down-regulation compared with wild-type Nef, which ranged from 8 to 20 times between experiments.

Endocytosis and recycling experiments

The FACS-based endocytosis assay (Chambers *et al*., 1993) was described previously (Mangasarian *et al*., 1997). Briefly, 10^7 cells were incubated with a phycoerythrin-conjugated monoclonal antibody specific for CD4 or CD8 for 60 min in DMEM containing 2% FCS at 4°C. Following two washes to remove unbound antibody, total cell-associated antibody was measured by FACS by transferring 1.5×10^6 cells in 50 μl into a 7-fold excess of PBS. Cells were incubated at 37°C and, at appropriate time points, 50 μl aliquots were placed in an ice-cold buffered saline solution at pH 2 and analyzed by FACS after 45 s. The fractions of internalized CD4- and CD8-derived molecules were calculated as previously described (Mangasarian *et al*., 1997) by subtracting the mean fluorescence of the initial time zero acid wash (measured before warming the cells) from all values and then dividing the mean fluorescence values after acid wash (internalized) for each time point by the total antibody (surface plus internalized) mean fluorescence. Endocytosis assays performed using ^{125}I-labeled antibodies (Pelchen-Matthews *et al*., 1989, 1991; Aiken *et al*., 1994) gave results similar to those obtained with the FACS-based method. It was also shown previously that Fab fragments and divalent antibodies can be used interchangeably to study CD4 endocytosis (Pelchen-Matthews *et al*., 1989, 1991). To test the dominant-negative phenotype of SIV Nef mutants, 293T cells were transiently transfected with 3 μg of the CD4-expressing CMX-CD4 vector (Aiken *et al*., 1994), together with either the CMX-PL1 empty vector (Umesomo *et al*., 1991), the wild-type HIV-1 and SIV Nef-producing constructs CMX-Nef1 or CMX-NefSIV_{MAC239} (9 μg) or a mix of wild-type SIV or HIV-1 Nef and SIV Nef mutant plasmids at a ratio 1:1 or 1:3 (9 μg of wild-type and 9 μg of mutant, or 9 μg of wild-type and 27 μg of mutant SIV Nef). All transfections were normalized by transfecting appropriate amounts of the CMX-PL1 empty vector. Recycling experi-

ments were performed as previously described (Pelchen-Matthews *et al*., 1989), with slight modifications. Cells were labeled as for endocytosis studies, incubated at 37°C for 30 min to allow the antibody to be internalized, placed at 4°C, washed in acid medium pH 3 twice for 2 min and analyzed by FACS. Cells were then rewarmed for the indicated times, washed again in cold acid and fluorescence was measured by FACS. The percentage of CD4 recycled was determined by dividing the remaining mean fluorescence after rewarming by the mean fluorescence after the first warming at 37°C for 30 min. Fluorescence was stable during the rewarming times as measured by FACS by placing the cells in PBS at pH 7 during the rewarming steps instead of the final acid wash. The viability of the cells after acid treatment before rewarming was determined both by staining with propidium iodide and by measuring the capacity of these cells to internalize a conjugated antibody. Dead cells were excluded by FSC and SSC on the FACS. Typically ~90% of the gated cells were alive throughout the experiment.

Immunoelectron microscopy

Transiently transfected 293T cells expressing either 88SIVNef$_{1–59}$ or 88SIVNef$_{1–59YY}^{28/39}{}_{GG}$ were removed from dishes 2 days after transfection with 2 mM EDTA in PBS, washed and resuspended in PBS/BSA 1% (10^7 cells/ml). Cells were then incubated for 2 h at 4°C with a primary mouse anti-CD8 antibody (RPAT8, Pharmingen). After antibody binding, cells were washed twice by centrifugation (200 *g* for 5 min) and incubated further for 1 h at 4°C with an anti-mouse IgG coupled to 10 nm colloidal gold particles (Sigma) diluted 1:15 in PBS/BSA 1%. After two washes in cold PBS to remove unbound secondary antibody, cells were warmed for 5 min at 37°C to allow the active association of the gold complex with CCPs. Cells were then fixed for 30 min at room temperature with 2.5% glutaraldehyde in phosphate buffer (pH 7.4), dehydrated and processed for electron microscopy. Thin sections were examined in a Philips EM 301, and gold particles were analyzed quantitatively. For each time point studied, for each cell line, ~3700–5000 gold particles were analyzed from cells expressing the chimeric molecules (i.e. cells presenting a specific gold labeling) and judged to be morphologically well preserved. Gold particles were scored in terms of their association with uncoated or coated domains of the membrane. They were considered associated with clathrin-coated structures when they were observed immediately adjacent (at a distance <20 nm) to the clathrin coat or totally enclosed in clathrin-coated pits/vesicles, as previously described (Foti *et al*., 1997; Mangasarian *et al*., 1997). Results were expressed as the percentage of the total number of surface-associated gold particles that were present in CCPs.

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