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Nef Interacts with the μ Subunit of Clathrin Adaptor Complexes and Reveals a Cryptic Sorting Signal in MHC I Molecules

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Summary

The surface expression of MHC I is reduced in HIV-infected cells. We show that the Nef protein affects the intracellular sorting of HLA-A and -B molecules. In the presence of Nef, these proteins accumulate in the Golgi and colocalize with clathrin-coated vesicles. MHC I modulation relies on a tyrosine-based sorting signal located in the cytoplasmic domain of HLA-A and -B heavy chains. This cryptic sorting signal becomes operative only in the presence of Nef. Nef interacts with the medium (μ) subunit of AP adaptor complexes involved in the recognition of tyrosine-based sorting signals, likely facilitating the connection between MHC I and the clathrin-dependent sorting machinery.

Introduction

Nef is a 27–30 kDa myristoylated protein encoded by primate lentiviruses that is produced in abundance at an early stage of the viral cycle (reviewed by Harris, 1996). Nef is essential for high-titer replication of SIV (simian immunodeficiency virus)mac239 and disease induction in macaques (Kestler et al., 1991). Nef is expressed in HIV (human immunodeficiency virus)-infected patients, and *nef*-defective viral strains have been implicated in some cases of long term survival of infection. Molecular mechanisms supporting the biological effects of Nef are not fully elucidated. Several properties have been attributed to Nef. First, Nef affects signal transduction and activation pathways in T cells, probably through its association with cellular kinases. Second, Nef facilitates reverse transcription in the incoming virion, thus enhancing virus particle infectivity. Third, Nef down-regulates cell surface CD4 and major histocompatibility class I (MHC I) molecules, which accumulate intracellularly and are degraded in the endosomal pathway (Garcia and Miller, 1991; Aiken et al., 1994; Schwartz et al., 1995a, 1996). This effect has been attributed to a stimulation by Nef of the endocytosis of surface molecules (Aiken et al., 1994; Schwartz et al., 1995a, 1996). However, the retention of neosynthetized CD4 molecules in the Golgi also contributes to the modulation (Mangasarian et al., 1997). The intracellular retention of CD4 is mediated by a dileucine signal located in the cytoplasmic domain (Aiken et al., 1994). The surface expression of other integral membrane proteins tested is not affected by Nef.

A variety of leucine-based and tyrosine-based motifs (LZ and YXXZ/NXXY respectively, where L indicates a leucine, Y a tyrosine, X any amino acid [aa], and Z one of the hydrophobic aa [L, I, V, M, F, C, or A]) located in the cytoplasmic domain of integral membrane proteins function as sorting signals for the endosomal pathway. Selection may occur in the *trans*-Golgi network (TGN) or at the plasma membrane (Sandoval and Bakke, 1994; Marks et al., 1997). The γ and δ chains of the T cell receptor, the MHC II associated invariant chain, the epidermal growth factor (EGF) receptor, and the CD4 molecule bear a leucine-based motif (Sandoval and Bakke, 1994; Marks et al., 1997). Tyrosine-based motifs are present in a number of molecules including the low density lipoprotein, EGF, mannose-6-phosphate, and transferrin receptors (Trowbridge et al., 1993; Marks et al., 1997). Tyrosine-based motifs are recognized by the adaptor protein (AP) complexes (Robinson, 1994; Ohno et al., 1995). Association of AP complexes with cytosolic clathrin induces the budding of clathrin-coated vesicles. At least two different but related AP complexes exist in the cell, one associated with the plasma membrane (AP-2) and one with the TGN (AP-1). The plasma membrane AP-2 complexes are heterotetramers with two large proteins of 100 kDa (α-adaptin and either β2- or β1-adaptin), a 50 kDa medium chain (μ2), and a 17 kDa small chain (σ2). The TGN AP-1 complexes associate the γ and β1- or β2- adaptins, the 47 kDa μ1 medium chain, and the 19 kDa σ1 small chain. The μ1 and μ2 subunits bind directly to tyrosine-based signals of sorted proteins (Ohno et al., 1995; Marks et al., 1997). Conformational changes leading to exposure of the tyrosine residue may be required for recognition of the sorting signal (Trowbridge et al., 1993; Sandoval and Bakke, 1994).

MHC I proteins exist in a highly polymorphic, membrane-anchored heavy chain, which is noncovalently associated with β_2-microglobulin. The assembly of the heavy chain with β_2-microglobulin takes place in the endoplasmic reticulum or *cis*-Golgi apparatus, where they are associated with antigenic peptides (Bijlmakers and Ploegh, 1993). The trimolecular complexes are then addressed to the cell surface (Neefjes et al., 1990). Heavy chains are formed of three extracellular domains, a transmembrane domain encoded by exon 5, and a 34 aa cytoplasmic tail encoded by exons 6, 7, and 8. MHC I complexes are stably expressed at the cell surface with only a minor fraction of molecules being internalized spontaneously in T cells and in monocytes/macrophages (Neefjes et al., 1990; Reid and Watts, 1990).

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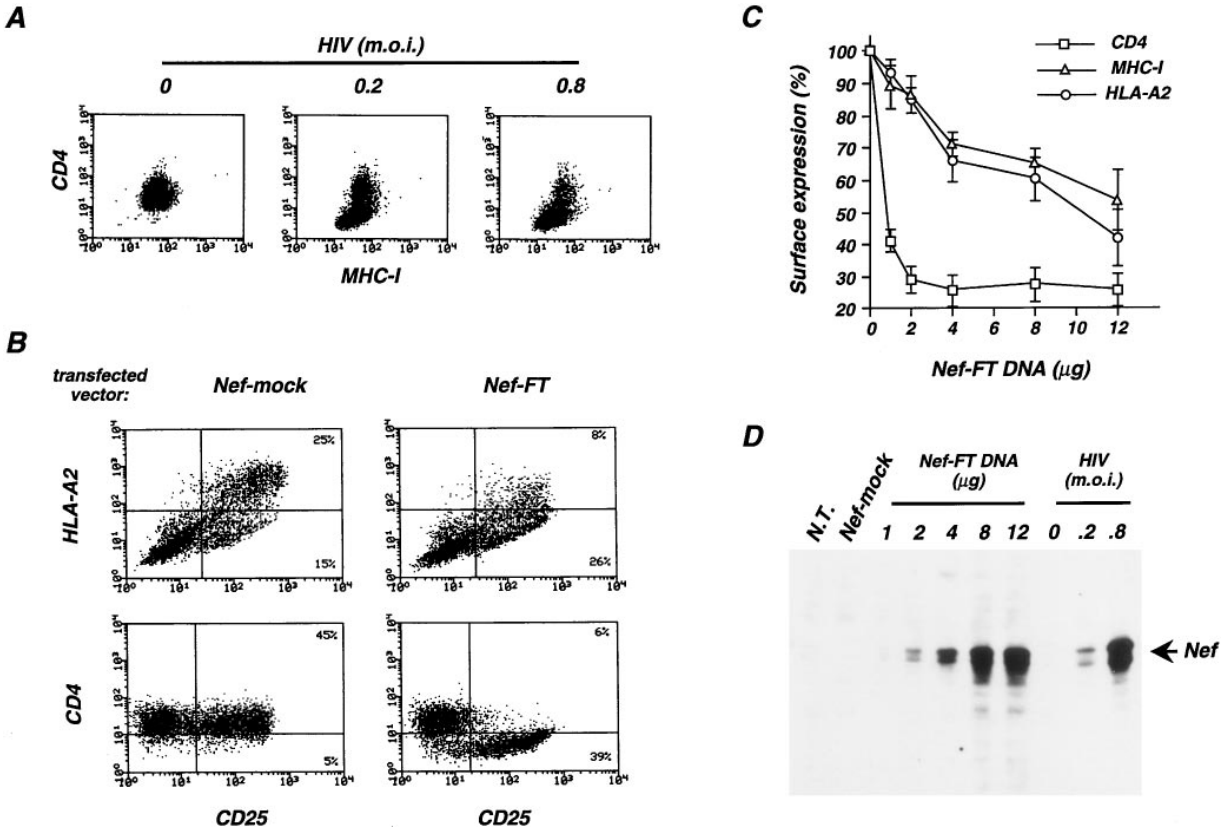


Figure 1. Nef-Induced Modulation of CD4 and MHC I in HeLa-CD4 Cells

Two-color flow cytometry analysis of HeLa-CD4 cells infected by HIV-1 (A) or transiently transfected (B and C).

(A) Cells were infected with HIV-1 (NL43) at the indicated multiplicity of infection (moi). Cells were doubly stained 24 hr later with anti-CD4 (Leu3a) and anti-MHC I (W6/32) mAbs and analyzed by flow cytometry.

(B) Cells were cotransfected with 12 μg of the Nef-FT vector or the Nef-mock plasmid, 4 μg of the CD25, and 4 μg of the A2 WT vectors. Cells were doubly stained 24 hr later with anti-CD25 (33B3) and HLA-A2 (BB7.2) mAbs (top), or with anti-CD25 and anti-CD4 mAbs (bottom) and analyzed by flow cytometry. Percentages of high and low HLA-A2 and $CD4^+$ cells among $CD25^+$ cells are indicated.

(C) Dose-dependent CD4 and MHC I modulation in response to Nef. HeLa-CD4 cells were coelectroporated with 4 μg of the A2 WT vector, 4 μg of the CD25 vector, and the indicated amounts of Nef-FT vector. Cells were doubly stained 24 hr later with the anti-CD25 (33B3) and anti-CD4 (Leu3a) mAbs, the anti-CD25 (IL2R-PE) and anti-MHC I (W6/32) mAbs, or with the anti-CD25 (33B3) and anti-HLA-A2 (BB7.2) mAbs and then analyzed by flow cytometry. The mean of CD4, HLA-A2, and total MHC I fluorescence in $CD25^+$ cells is shown on the ordinate, where 100% values correspond to the fluorescence level of cells transfected with the control Nef-mock plasmid. Data are means ± SD of three independent experiments.

(D) Western blot analysis. Lysates from cells infected with HIV-1 or from transfected cells (Figures [A] and [C], respectively) were analyzed with an anti-Nef mAb (MATG 020). Nef-mock: cells were transfected with 12 μg of the Nef-mock plasmid. NT, nontransfected control cells. Data are representative of three independent experiments.

Signals mediating spontaneous endocytosis have been localized in the cytoplasmic domain encoded by exon 7 (Vega and Strominger, 1989). Leucine- or tyrosine-based motifs are not found in this domain.

We reported previously that MHC I proteins are normally synthetized and transported through the endoplasmic reticulum and *cis*-Golgi apparatus in Nef-expressing cells, whereas surface MHC I molecules are targeted to endosomes and degraded (Schwartz et al., 1996). We show that Nef acts additionally by misrouting MHC I to clathrin-coated vesicles budding from the TGN. Nef binds to μ1 and μ2 medium chains of AP complexes and reveals a cryptic tyrosine-based motif located in HLA (human leukocyte antigen)-A and -B cytoplasmic domains. HLA-C molecules do not bear this motif and are not affected by Nef. Our data suggest that the recognition of this signal by the clathrin-dependent cell-sorting machinery accounts for the addressing of HLA-A and -B molecules from both the TGN and the plasma membrane to the endosomal compartments.

Results

Nef-Induced MHC I Down-Regulation

MHC I surface expression levels are reduced in U937 monocytic or CEM lymphoblastoid cells infected with HIV-1 or stably expressing HIV-1 *nef* alleles (Schwartz et al., 1996). To address the molecular basis of the Nef-mediated MHC I modulation, we designed a transient transfection assay in HeLa-CD4 cells. We verified that HIV-1 infection of HeLa-CD4 cells induced the down-regulation of surface MHC I. Cells were infected with HIV-1 (multiplicity of infection = 0.2 or 0.8) and doubly stained 24 hr later with anti-CD4 and anti-MHC I monoclonal antibodies (mAbs) (Figure 1A). Flow cytometry analysis revealed a CD4-low, MHC I–low cell population,

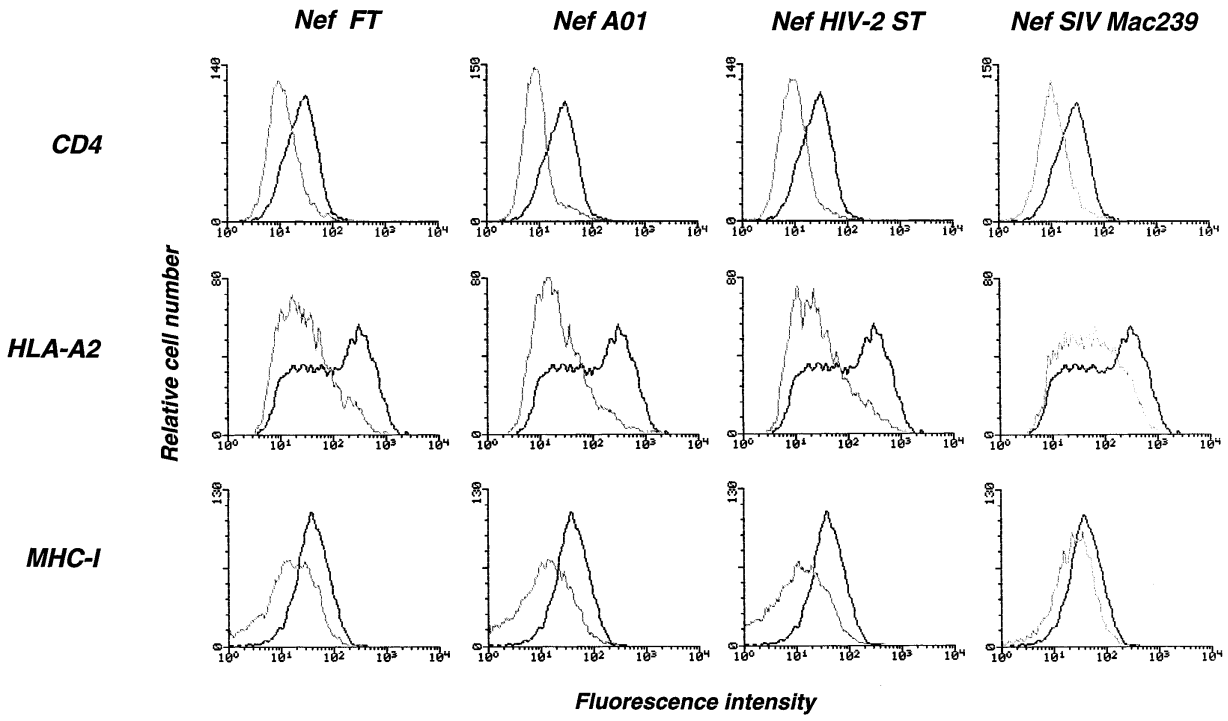


Figure 2. Modulation of CD4 and MHC I by Nef Proteins from HIV-1, HIV-2, and SIV

HeLa CD4 cells were transfected with 12 μg of the Nef-FT, Nef A01, Nef HIV-2, or Nef SIV vectors (gray curves) or with 12 μg of the Nef-mock plasmid (black curves), along with 4 μg of the CD25 and A2 WT vectors. After 24 hr, surface expression of CD4 (top row), HLA-A2 (middle row), and total MHC I (bottom row) was analyzed in $CD25^+$ cells by flow cytometry. Data are representative of three independent experiments.

the size of which correlated with the viral input (Figure 1A). Therefore, HIV-1 infection of HeLa-CD4 cells induces MHC I down-regulation.

Expression vectors were constructed in which the HIV-1LAI *nef* gene (Nef-FT vector), an anti-sense *nef* gene (Nef-mock vector), the HLA-A2 cDNA (A2 wild-type [WT] vector), or the human CD25 cDNA (CD25 vector) were inserted downstream of the CMV (cytomegalovirus) promoter. HeLa-CD4 cells, which are HLA-A2 negative, were cotransfected with CD25, A2 WT, and Nef-mock or Nef-FT vectors. Transfection efficiencies were in the range of 30%–50% as shown by the analysis of CD25 surface expression 24 hr post-transfection (Figure 1B). As expected, cotransfection of Nef-FT decreased CD4 levels, whereas CD25 expression was not affected. HLA-A2 surface levels were reduced in $CD25^+$ cells transfected with the Nef-FT vector, confirming that Nef induced a modulation of MHC I in HeLa-CD4 cells. A similar observation was made in HeLa cells lacking CD4, indicating that modulation of MHC I was not a result of that of CD4 (data not shown).

The extent of MHC I modulation was examined for various levels of Nef expression (Figure 1C). The modulation of HLA-A2 increased with the amount of transfected Nef-FT vector, reaching a 60% reduction with 12 μg of DNA. Staining of the cells with the anti-HLA-A, -B, -C mAb W6/32, which recognizes both endogenous MHC I and transiently expressed HLA-A2 molecules, showed a similar decrease of MHC I surface expression. Therefore, both endogenous and transiently expressed MHC I molecules were affected by Nef. Transfection of 12 μg of CMV vectors expressing either an inactive (nonmyristoylated) form of Nef or the β-galactosidase (β-gal) protein indicated that the transient overexpression of a control protein does not affect MHC I surface levels (data not shown). Western blot analysis showed that levels of Nef expression were proportional to the amount of transfected Nef-FT vector (Figure 1D). In cells transfected with 12 μg of vector DNA, the Nef signal was equivalent to that detected in HIV-infected HeLa-CD4 cells. Therefore, the levels of Nef expression required to mediate MHC I down-regulation in the transfection assay were within the range of those produced in infected cells. The levels of CD4 surface expression were also examined for various amounts of Nef-FT (Figure 1C). A maximal 75% decrease was observed with 2 μg of transfected DNA, indicating that CD4 was more susceptible than MHC I to the modulation induced by Nef.

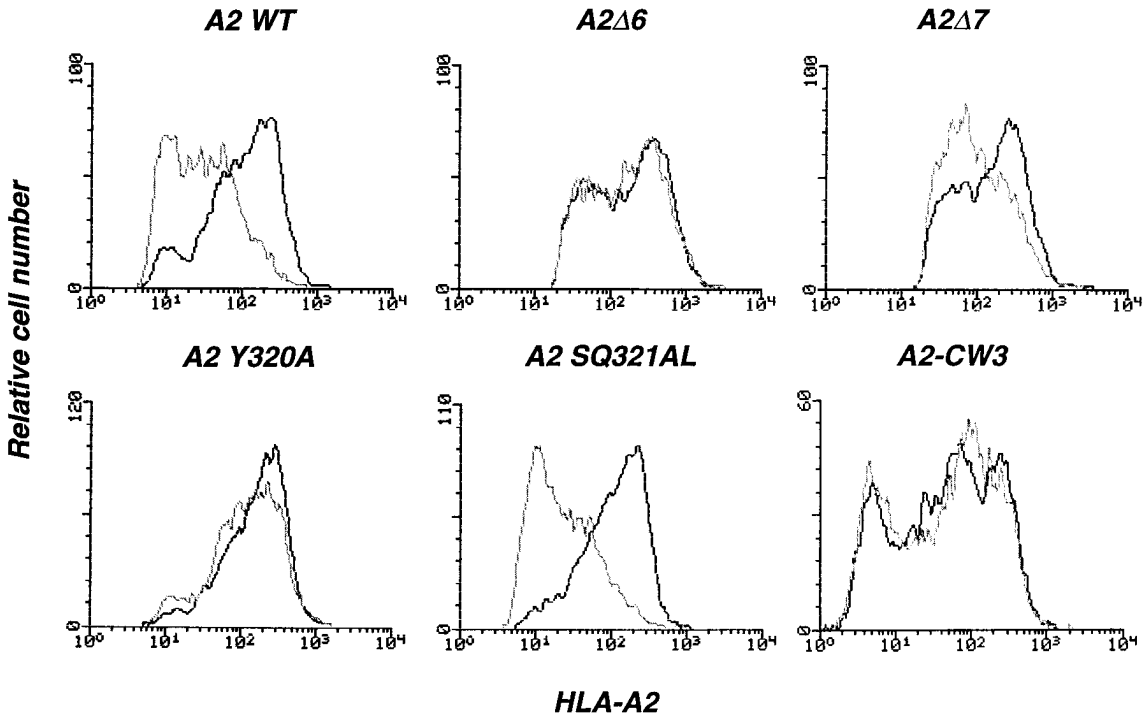
Down-Regulation of Cell Surface CD4 and MHC I by Nef Proteins from Various Primate Lentiviruses

CMV expression vectors coding for the Nef proteins of a primary HIV-1 isolate (Nef-A01) (Schwartz et al., 1993), HIV-2ST (Nef-HIV2), or SIVmac239 (Nef-SIV) (Aiken and Trono, 1995) were constructed and their effect on MHC I expression was examined after transfection of HeLa-CD4 cells. As a control of Nef activity, CD4 surface expression was analyzed in $CD25^+$ cells by flow cytometry (Figure 2, top row). As expected, CD4 modulation was induced by the various Nef molecules. Nef-FT, Nef-A01, and Nef-HIV2 reduced HLA-A2 surface expression equally, whereas Nef-SIV was slightly less efficient (Figure 2, middle row). A similar observation was made by

A

Coding sequence:	TM Exon 5	INTRACYTOPLASMIC DOMAIN Exon 6	Exon 7	Exon 8
	310	320	330	340
A2 WT	RRKSS	DRKGGSYSQAA	GSDSAQGSDVSLTACK	V
A2Δ6	RRKSS	----------	GSDSAQGSDVSLTACK	V
A2Δ7	RRKSS	DRKGGSYSQAA	----------	V
A2 Y320F	RRKSS	DRKGGS**F**SQAA	GSDSAQGSDVSLTACK	V
A2 Y320A	RRKSS	DRKGGS**A**SQAA	GSDSAQGSDVSLTACK	V
A2 SQ321AL	RRKSS	DRKGGSY**AL**AA	GSDSAQGSDVSLTACK	V
HLA-A consensus	RRKSS	DRKGGSYSQAA	SSDSAQGSDVSLTACK	V
HLA-B consensus	RRKSS	**GG**KGGSYSQAA	SSDSAQGSDVSLTACK	V
HLA-C consensus	RRKSS	**GG**KGGS**C**SQAA	SS**N**SAQGSD**E**SL**I**ACK	**A**

B



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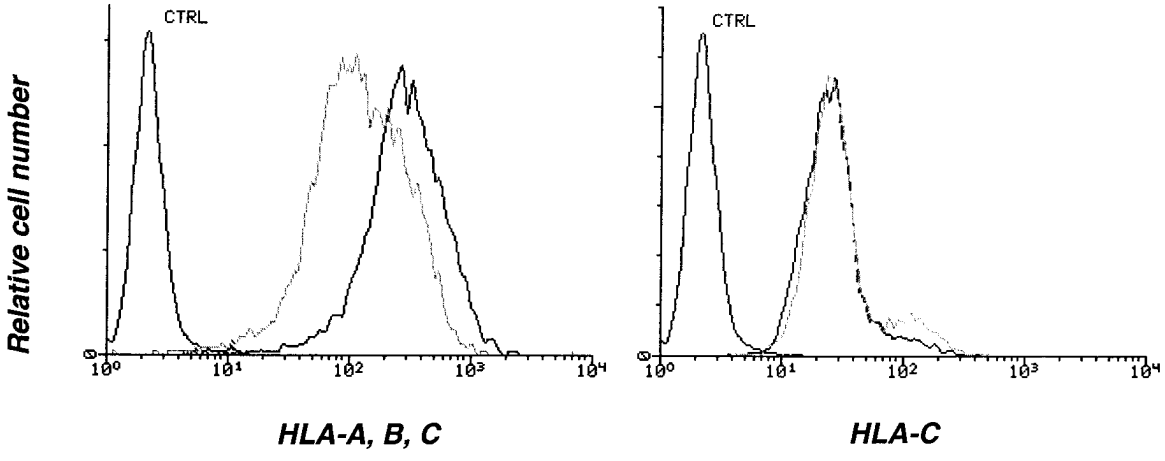

staining the cells with the polymorphic anti-HLA mAb W6/32 (Figure 2, bottom row). Therefore, the modulation of MHC I molecules is a property shared by different biologically active Nef isolates from primate lentiviruses.

A Tyrosine-Based Motif Is Required for Nef-Induced MHC I Down-Regulation

We examined which determinants of MHC I are required for Nef effect. Mutants of the HLA-A2 HC were constructed in which the regions of the cytoplasmic tail encoded by the exon 6 or 7 of the *HLA-A2* gene were deleted (Figure 3A). Modified genes were introduced in CMV expression vectors (A2-Δ6 and A2-Δ7 vectors). Flow cytometry analysis of HeLa cells transfected with the Nef-mock vector revealed equivalent cell surface expression of WT and deleted HLA-A2 molecules (Figure 3B). Transfection of Nef-FT decreased the cell surface level of HLA-A2 and A2-Δ7 but did not affect that of A2-Δ6. Therefore, the determinants required for Nef-induced modulation are located in the cytoplasmic domain of HLA-A2 encoded by exon 6.

The polypeptide sequence encoded by exon 6 is highly conserved between HLA alleles (73%–90% homology). Tyrosine residue 320 is present in all HLA-A and -B molecules (Parham et al., 1995). The sequence YSQA (residues 320–323) is reminiscent of tyrosine-based motifs that mediate endocytosis and sorting of a number of surface molecules. We asked whether tyrosine 320 of HLA-A2 is important for Nef-induced down-regulation. This residue was exchanged for an alanine or a phenylalanine (mutants A2-Y320A and A2-Y320F, respectively) and a control mutant was constructed in which adjacent serine 321 and glutamine 322 were exchanged for alanine and leucine, respectively (A2-SQ321AL) (Figure 3A). In the absence of Nef, WT and mutant HLA-A2 molecules were expressed equally at the cell surface (Figure 3B). Transfection of Nef-FT decreased surface level of A2 WT and A2-SQ321AL but did not affect that of A2-Y320A (Figure 3B) and A2-Y320F (data not shown). Therefore, the tyrosine 320 residue is necessary for Nef-induced HLA-A2 modulation. It is presumable that in the presence of Nef the YSQA sequence functions as a tyrosine-based motif mediating the modulation of MHC I molecules. In vivo, HLA-A2 molecules are phosphorylated on serine 335, which is encoded by exon 7, but not on tyrosine 320 (Guild and Strominger, 1984). Consistently, we found that A2-Δ7, which does not bear the serine 335 but still contains tyrosine 320, was not phosphorylated. Coexpression of Nef did not induce the phosphorylation of A2-Δ7, indicating that Nef action is not mediated by a phosphorylation of tyrosine 320 (data not shown).

We verified that HLA-B molecules were also modulated by Nef. HeLa cells, which are HLA-B7 negative, were transfected with a HLA-B7 expression vector and analyzed as previously with an anti-B7 mAb. Nef induced the modulation of surface HLA-B7 (not shown). Modulation of HLA-B was confirmed in CEM cells by using the B1.23.2 mAb, which recognizes HLA-B and C molecules (Ferrier et al., 1985). A significant decrease of surface staining was observed in CEM cells stably expressing different Nef proteins (Schwartz et al., 1993) (data not shown).

HLA-C Surface Expression Is Not Affected by Nef

HLA-C molecules carry a cysteine at position 320 instead of a tyrosine (Parham et al., 1995) (Figure 3A). HLA-C molecules are dominant inhibitory ligands protecting target cells against lysis by natural killer lymphocytes (NK) (Brutkiewicz and Welsh, 1995). We examined whether Nef affects HLA-C surface expression. HeLa cells were cotransfected with the CD25 and the Nef-FT or the Nef-mock vectors. Cells were stained with either the W6/32 polymorphic anti-HLA mAb or with the L31 mAb, which is specific for HLA-C heavy chains (Ciccone et al., 1995). The surface expression of total MHC I molecules was reduced in the presence of Nef, whereas HLA-C was expressed equally with or without the viral protein (Figure 3C). We also constructed a vector encoding a chimeric protein associating the extracellular and transmembrane domains of HLA-A2 with the cytoplasmic tail of HLA-Cw3 (A2-CW3) and observed that A2-CW3 was not modulated by Nef (Figure 3B). The absence of a modulation of HLA-C molecules in the presence of Nef confirms that tyrosine 320 is required for Nef action.

MHC I Molecules Colocalize with Clathrin and AP Complexes in the Presence of Nef

We examined the subcellular localization of MHC I in the presence of Nef. Cells were transfected with the HLA-B7, A2 WT, or A2-Y320A vectors, along with the Nef-FT or Nef-mock plasmids. The subcellular localization of HLA-B7 or -A2 was examined 24 hr later (Figure 4A). An intense cell surface staining and a weak intracellular signal were detected for B7, A2 WT, and A2-Y320A

Figure 3. Susceptibility of HLA-A2 Mutants and HLA-C to Nef-Induced Modulation

(A) Amino acid sequence alignment of the WT (A2 WT), the deletion and substitution mutants of the cytoplasmic domain of HLA-A2, and of the consensus sequences for HLA-A, -B and -C. Dashes indicate deleted regions and bold letters identify aa substitutions in the single-letter code. The nomenclature of mutant HLA-A2 proteins is indicated on the left side of the panel. Consensus sequences are from Parham et al. (1995) and aa numeration is according to Vega and Strominger (1989).

(B) Surface levels of the WT and mutant HLA-A2 molecules in the absence or in the presence of HIV-1 Nef. HeLa cells were electroporated with 12 μg of Nef-FT (gray curves) or Nef-mock vector (black curves), along with 4 μg of the A2 WT or mutant vectors and with 4 μg of the CD25 vector. After 24 hr, surface expression of HLA-A2 was analyzed in $CD25^+$ cells by flow cytometry. A2-CW3 is a chimeric protein associating the extracellular and transmembrane domains of HLA-A2 with the cytoplasmic tail of HLA-Cw3.

(C) Surface levels of total MHC I and of HLA-C molecules in the absence or presence of Nef. HeLa cells were electroporated with 12 μg of Nef-FT (gray curves) or Nef-mock vector (black curves) and 4 μg of CD25 vector. After 24 hr, surface expression of total MHC I and of HLA-C was analyzed in $CD25^+$ cells by using the W6/32 and L31 mAbs, respectively. CTRL, cells stained with secondary antibodies, as a negative control. Data are representative of three experiments.

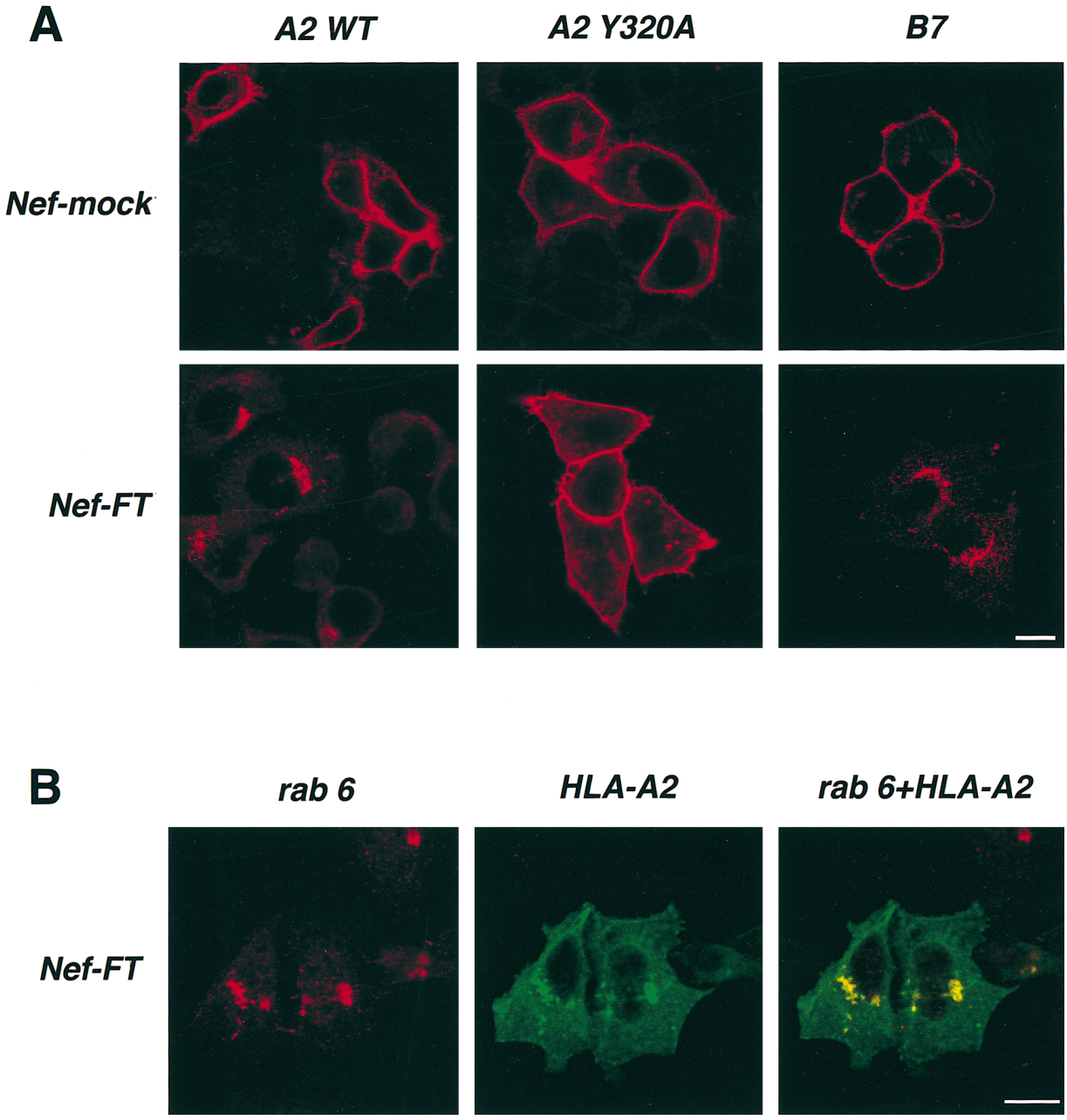

Figure 4. Intracellular Retention of MHC I Induced by Nef

(A) HeLa cells were transfected with 12 μg of the Nef-mock (top) or Nef-FT (bottom) plasmid, along with 4 μg of the A2 WT (left), A2 Y320A (middle), or HLA-B7 (right) vectors. After 24 hr, cells were fixed, permeabilized, and stained with an anti-HLA-A2 (BB7.2) or anti-HLA-B7 mAb (BB7.1). The localization of HLA-A2 or -B7 was examined by immunofluorescence staining and confocal microscopy analysis. Transfected cells could be easily distinguished from untransfected cells by the simultaneous expression of HLA-A2 or -B7 and Nef (data not shown). Series of optical sections at 0.5 mm intervals were recorded. A representative medial section is shown.

(B) Simultaneous detection of Rab6 (a marker of the Golgi) and of HLA-A2 in Nef-expressing cells. Cells were doubly stained with an anti-HLA-A2 mAb (BB7.2) (green) and with rabbit anti-Rab6 (red) antibodies and analyzed as described in (A). The right column is a superposition of the two stainings, in which costained regions appear in yellow. Scale bar, 10 μm.

molecules in the absence of Nef. HLA-B7 and -A2 surface stainings were strongly reduced in Nef-expressing cells. Cytoplasmic dots, located mostly in the perinuclear region and also at the margin of the cells, were observed. In agreement with flow cytometry data, the surface localization of A2-Y320A was not affected by Nef (Figure 4A). Nef-expressing cells were then doubly stained with antibodies against HLA-A2 and Rab6, a marker of the medial and *trans*-Golgi (Goud et al., 1990) (Figure 4B). MHC I and Rab6 stainings overlapped in the perinuclear region. Few vesicles located at the vicinity of the Golgi and at the cell periphery contained MHC I but not Rab6. Therefore, in the presence of Nef, an important fraction of MHC I molecules is located in the Golgi area.

Tyrosine-based motifs located in the cytoplasmic domain of membrane proteins mediate clathrin-dependent

sorting to the endocytic pathway from both the TGN and the plasma membrane. We examined the subcellular localization of MHC I concurrently with those of clathrin or AP complexes. HeLa cells were transfected with the HLA-B7 vector along with Nef-FT or Nef-mock plasmids and stained 24 hr later. In control cells, labeling with anticlathrin antibodies showed typical bright perinuclear staining and discrete peripheric dots, corresponding to vesicles budding from the Golgi and the plasma membrane, respectively (Figure 5A) (Robinson, 1987). AP-1 complexes were visualized with anti-γ adaptin mAbs. A punctate perinuclear staining extending to the periphery was observed (Robinson, 1993) (Figure 5B). Without Nef, MHC I molecules were found mostly at the cell surface, with a weak intracellular signal in the Golgi area that likely corresponded to newly synthesized molecules engaged along the secretory pathway. Very little of MHC I staining colocalized with that of clathrin or γ adaptin. Nef did not significantly modify the subcellular localization of clathrin and γ adaptin (Figure 5). In Nef-expressing cells, the MHC I signal overlapped with those of clathrin and γ adaptin, indicating that a significant fraction of MHC I was located in the same area as clathrin-coated vesicles bearing AP-1 complexes and budding from the TGN. We previously showed that prior steps of the transport of neosynthesized MHC I molecules through the endoplasmic reticulum/*cis*-Golgi compartments are not affected by Nef (Schwartz et al., 1996). Several lines of evidence suggest that the perinuclear localization of HLA-A2 or -B7 is not a consequence of the accumulation of transiently overexpressed proteins in the secretory pathway. Cotransfection of HLA-A2 or -B7 and Nef-mock vectors did not induce the intracellular accumulation of MHC I (Figures 4 and 5). Moreover, in Nef-expressing cells mutant A2 Y320A molecules were localized at the cell surface and were barely detected in the vicinity of the TGN (Figure 4). Altogether, these experiments suggest that Nef is responsible for the accumulation of HLA-A and -B in vesicles budding from the Golgi and operates on the mechanisms that mediate the recognition of the tyrosine-based signal of MHC I by AP complexes.

Nef Binds to μ1 and μ2 Subunits of AP Complexes

Because Nef may influence the recognition of a sorting signal in MHC I by the medium chains of AP complexes, we attempted to reveal interactions between AP complexes and Nef and between AP complexes and HLA-A2. Evidence for the presence of Nef in clathrin-coated vesicles containing HLA-A2 could not be obtained by colocalization experiments because of the widespread presence of Nef in subcellular compartments (data not shown). Interactions were therefore investigated using yeast two-hybrid and cell-free assays.

Interaction of Nef with the components of AP-1 and AP-2 complexes was first examined. Nef proteins from HIV-1Lai, HIV-2Rod, and SIVmac239 were fused to the Gal4 DNA binding domain (Gal4BD), and interaction with hybrids of μ1 or μ2 with the Gal4 activator domain (Gal4AD) was assayed. After transformation with the Gal4BD and Gal4AD hybrids, HF7c and SFY526 yeast reporter strains were assayed for histidine auxotrophy and for β-gal expression, respectively. Growth of the HF7c strain in histidine-free medium indicated transactivation of the *HIS3* reporter gene, providing evidence for interaction between HIV-1LaiNef and μ1 hybrids (Figure 6A, lane 1). Similar results were obtained with *nef* alleles from HIV-1 primary isolates A01 and E01 and from the HXB3 HIV-1 laboratory strain (data not shown). A low β-gal activity was also revealed in the SFY526 strain (Figure 6A, left). Interaction was not detected between NefLai and μ2 hybrids (Figure 6A, lane 2). In contrast, Nef proteins from HIV-2Rod and SIVmac239 interacted with both μ1 and μ2 and induced a strong β-gal activity (Figure 6A, lanes 4 and 5 and lanes 7 and 8, respectively). Gal4BD-Nef hybrids did not bind to Gal4AD hybrids formed with the α, γ, β1, and β2 adaptins (data not shown). Therefore, in the two-hybrid assay Nef binds specifically to the medium chains and not to other components of AP complexes. N- and C-terminal deletion mutants of Nef were examined for μ1 binding. Results shown in Figure 6B indicate that a region encompassing aa 143–170 of NefLai supports interaction with μ1 in the two-hybrid assay. This region corresponds to a polypeptidic loop that is disordered in the crystal structure of the conserved core of Nef (Lee et al., 1996).

Interaction between Nef and μ chains was confirmed in cell-free assays. Recombinant HIV-1Lai and SIVmac239Nef fused to glutathione S-transferase (GST) were produced and immobilized on glutathione (GSH)-agarose beads. In vitro translated and [^{35}S] μ1 and μ2 proteins were tested for their capacity to bind immobilized GST-NefHIV-1Lai, GST-NefSIVmac239, or GST proteins. Bound labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and revealed by autoradiography. As shown in Figure 6C, [^{35}S]-μ1 and [^{35}S]-μ2 bound to GST-NefHIV-1Lai and GST-NefSIVmac239 but not to GST. We also asked whether Nef could interact with complete cellular AP complexes. HeLa-CD4 cells were lysed in a buffer preserving the integrity of AP complexes (Benmerah et al., 1996). Lysates were incubated with purified GST or GST-NefHIV-1Lai proteins immobilized on GSH-agarose beads. Bound proteins were analyzed by Western blotting with anti-γ (specific for AP-1) or with anti-β1+β2 adaptin (recognizing AP-1 and AP-2) mAbs. AP-1 complexes bound to GST-NefHIV-1Lai but not to GST (Figure 6D) nor to an irrelevant GST-USF fusion (data not shown). Altogether, yeast two-hybrid and cell-free binding studies indicate that Nef proteins from primate lentiviruses are capable of direct physical interactions with the μ1 and μ2 subunits of AP-1 and AP-2 complexes. Direct interaction of μ1 and μ2 with various tyrosine-based signals has been previously detected by using the two-hybrid system (Ohno et al., 1995; Boll et al., 1996). We could not detect interaction between the HLA-A2 cytoplasmic tail and μ1 or μ2 in the two-hybrid assay (data not shown), suggesting that in the absence of Nef the YSQA motif of HLA-A2 is recognized poorly or not at all by the medium chains.

Discussion

This work points out the interaction of Nef with the sorting machinery as a mechanism supporting the down-regulation of cell surface MHC I molecules. MHC I modulation is induced by the Nef proteins of HIV-1, HIV-2,

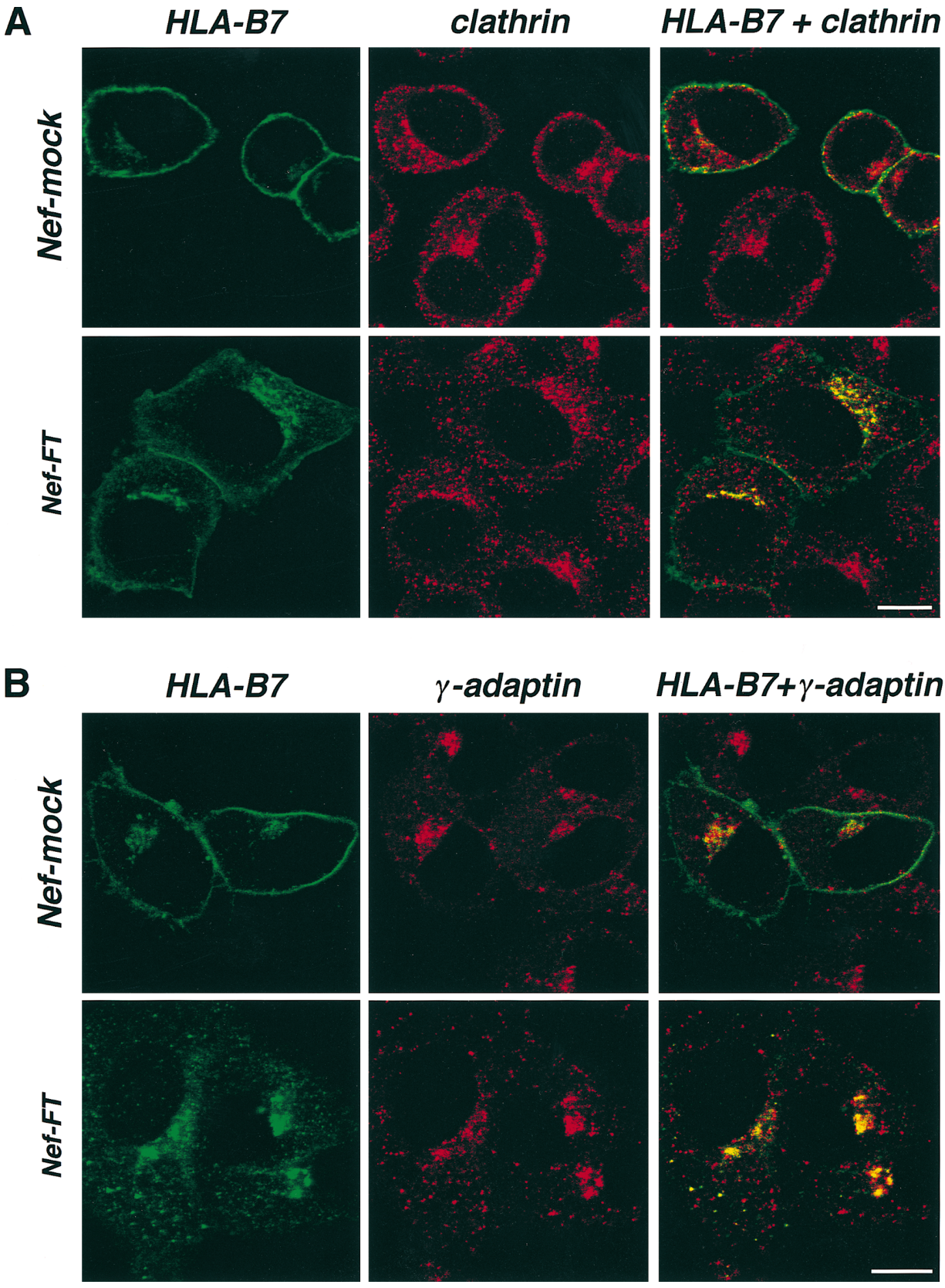

Figure 5. Localization of MHC I and Clathrin or AP-1 Complexes

Hela cells were electroporated with 12 μg of Nef-FT or Nef-mock and with 8 μg of the HLA-B7 vectors. After 24 hr, cells were doubly stained with an anti-HLA-B7 mAb (BB7.1) (green) and, in red, with either rabbit anti-clathrin antibodies (A) or anti-γ adaptin mAb (B). Cells were analyzed by confocal microscopy as described in the figure 4 legend. Scale bar, 10 μm.

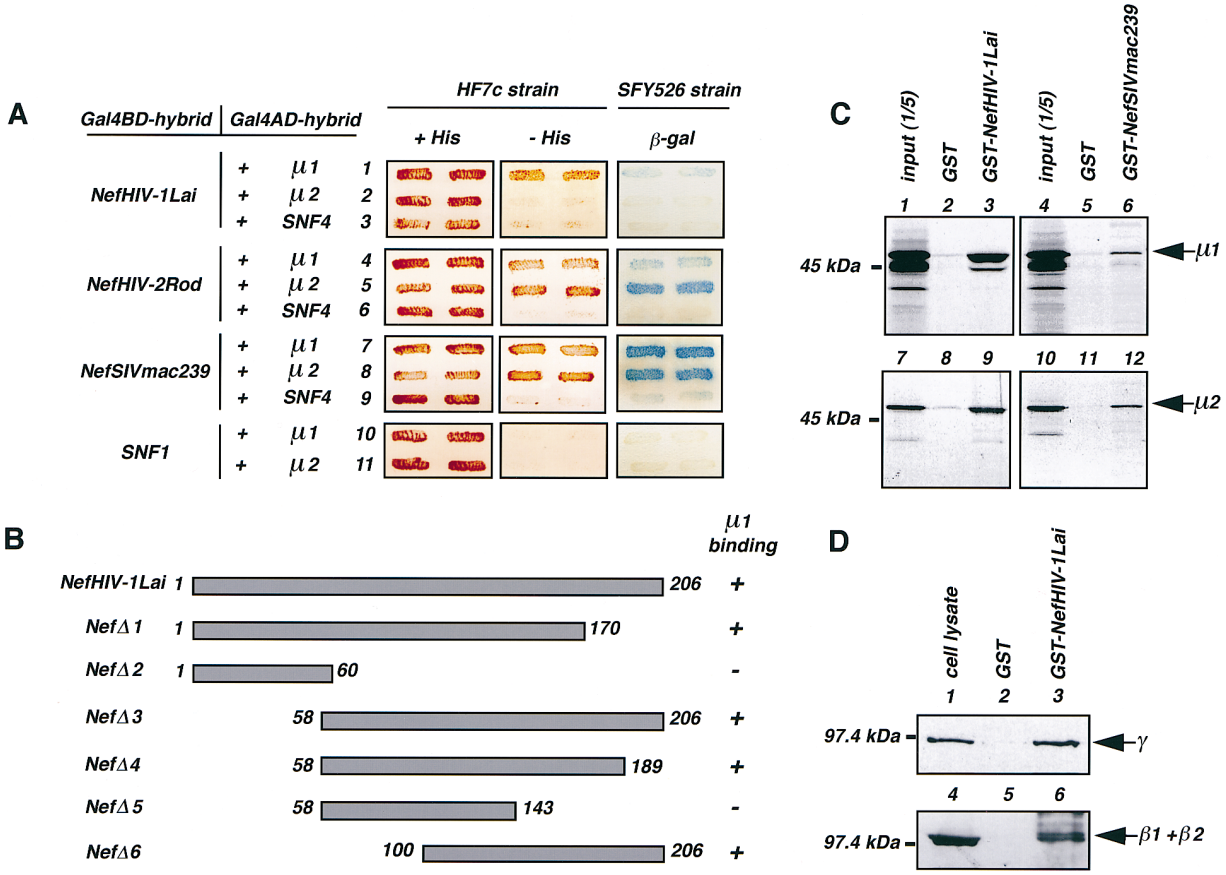

Figure 6. Binding of HIV and SIV Nef to μ1 and μ2 Subunits of AP Complexes

(A) Interaction of Nef with μ1 and μ2 in a yeast two-hybrid assay. HF7c and SFY526 yeast strains expressing the indicated pairs of Gal4BD and Gal4AD hybrid proteins were analyzed for histidine auxotrophy and β-gal activity, respectively. HF7c double transformants were patched on medium with histidine (left) and then replica-plated on histidine-free medium (middle). SFY526 double transformants were patched on selective medium and then replica-plated on Whatman filter for detection of β-gal activity (right). Growth in histidine-free medium and expression of β-gal indicate interaction between hybrid proteins. Yeast cells expressing the Gal4BD-NefHIV-1Lai (lanes 1–3), -NefHIV-2Rod (lanes 4–6), or -NefSIVmac239 (lanes 7–9) hybrids were analyzed. SNF1 and SNF4 yeast proteins, which bind to each other efficiently, were used as a positive control (data not shown). Binding specificity was verified by the absence of interaction between Nef and SNF4 (lanes 3, 6, and 9) and between SNF1 and μ (lanes 10 and 11). Each patch represents an independent transformant.

(B) Mapping of the NefHIV-1Lai domain interacting with μ1 in the two-hybrid assay. HF7c and SFY256 strains were cotransformed with Gal4AD-μ1 and each of the indicated Gal4BD-Nef mutant (NefΔ1–NefΔ6). N- and C- extremities of each Nef deletant are indicated by a number corresponding to the aa position in the Nef Lai sequence. Double transformants were analyzed for histidine auxotrophy and β-gal activity. + indicates transactivation of both reporter genes (*lacZ* and *HIS3*).

(C) Interaction of GST fusions of HIV-1Lai and SIVmac239 Nef with μ1 and μ2 proteins in a cell-free assay. [^{35}S]-μ1 (lanes 1–6) and [^{35}S]-μ2 (lanes 7–12) were synthetized in reticulocyte lysate and incubated with equal amounts of purified GST (lanes 2, 5, 8, and 11), GST-NefHIV-1Lai (lanes 3 and 9) or GST-NefSIVmac239 (lanes 6 and 12) previously immobilized on GSH-agarose beads. Bound labeled material was analyzed by SDS-PAGE and autoradiography. One fifth of the input of each in vitro translated product used for the binding assay was run on lanes 1, 4, 7, and 10.

(D) Binding of NefHIV-1Lai to complete cellular AP complexes. HeLa-CD4 lysates (from 25 × 10^6 cells) were incubated with equal amounts of purified GST (lanes 2 and 5) or GST-NefHIV-1Lai (lanes 3 and 6) previously immobilized on GSH-agarose beads. Bound proteins were resolved by SDS-PAGE and AP-1 association was analyzed by Western blotting with anti-γ (top) and anti-β1+β2 (bottom) adaptin mAbs. Crude lysates (from 10^6 cells) were also run as a control to detect γ and β adaptins (lanes 1 and 4, respectively). Data are representative of three independent experiments.

and SIV and has been observed in a variety of cell lines (Schwartz et al., 1996), indicating that various HLA-A and -B molecules are susceptible to Nef activity. Nef binds to the medium chains of AP complexes and reveals a cryptic tyrosine-based sorting signal in the cytoplasmic domain of HLA-A and -B heavy chains. HLA-C molecules, which do not bear the sorting signal, are not affected by Nef. Confocal microscopy analysis showed that in the presence of the viral protein, HLA-A and -B molecules accumulate in a perinuclear region that is stained by anti-clathrin, AP-1, and Rab6 antibodies, suggesting that Nef induces the accumulation of MHC I in clathrin-coated vesicles.

The effect of Nef on HLA-A and -B is reminiscent of the modulation of CD4, which is also induced by the viral protein. Electron microscopy studies documenting the role of Nef in the formation of clathrin-coated pits and in the recruitment of CD4 in these pits have been

reported (Foti et al., 1997; Mangasarian et al., 1997). Modulation of HLA-A and -B was observed in HeLa cells independently of the expression of CD4. Dose-response experiments in HeLa-CD4 cells indicated that CD4 modulation required lower amounts of Nef than the down-regulation of either exogenous or endogenous HLA-A and -B. The higher number of MHC I molecules than CD4 molecules at the surface of HeLa-CD4 cells and the absence of the p56^{lck} protein, which stabilizes CD4 at the cell surface (Pelchen-Mattews et al., 1993), may account for this difference.

The synthesis of HLA-A and -B is not affected by Nef, which reduces the half-life of the molecules at the cell surface, indicating that endocytosis accounts at least in part for MHC I modulation (Schwartz et al., 1996). We show that Nef acts at additional steps of MHC I sorting pathways. In the presence of Nef, MHC I molecules are located in the Golgi area, from which they are likely routed to the endosomal pathway and degraded (Schwartz et al., 1996). These observations, together with studies on Nef-induced CD4 modulation (Aiken et al., 1994; Schwartz et al., 1995a; Greenberg et al., 1997; Mangasarian et al., 1997) and evidence for the association of Nef with β-COP, a component of non–clathrin-coated vesicles (Benichou et al., 1994), indicate that Nef affects cellular sorting pathways. However, Nef operates specifically on CD4 and HLA-A and -B molecules. Other cell surface markers examined, including the receptors for EGF, low-density lipoprotein, transferrin, mannose-6 phosphate, and the CD8, CD20, CD25, CD69, and HLA-C molecules, are not affected by Nef.

Surface MHC I molecules are spontaneously internalized via coated pits in monocytes and T lymphocytes. This process is slow and involves a limited fraction of the surface molecules. Acceleration by T cell activation or by anti-MHC I antibodies has been reported (Vega and Strominger, 1989; Neefjes et al., 1990; Reid and Watts, 1990). MHC I molecules do not accumulate in the cytoplasm and most of them are recycled. Other endocytic pathways have been described, such as the antibody-induced internalization of surface MHC I via caveolae (Stang et al., 1997). By expressing HLA-A2 mutants in HeLa cells, we observed that Nef activity relies on the cytoplasmic domain encoded by exon 6. The determinants required for spontaneous endocytosis of MHC I molecules are encoded by exon 7 (Vega and Strominger, 1989). Therefore, spontaneous internalization and Nef-induced MHC I modulation involve different mechanisms. A tyrosine residue at position 320, which is present in HLA-A and -B but not in HLA-C heavy chains, is necessary for Nef activity. The sequence of the exon 6-encoded domain (DRKGGSYSQAA) suggests that tyrosine 320 may belong to a tyrosine-based sorting signal. Although tyrosine 320 is necessary for Nef activity, we cannot exclude the possibility that other elements are involved in the modulation.

Tyrosine-based sorting signals interact directly with the medium chains (μ1 and μ2) of clathrin-associated AP complexes and have been considered ligands for the sorting machinery (Ohno et al., 1995; Marks et al., 1997). At least two different AP complexes are involved in decoding sorting signals at the TGN (AP-1) and at the plasma membrane (AP-2) (Sandoval and Bakke, 1994). No direct interaction between μ1 or μ2 subunits and the cytoplasmic tail of HLA-A2 bearing the YSQA motif was observed using the two-hybrid system. It is therefore likely that AP complexes recognize the HLA-A2 tyrosine-based motif poorly in the absence of Nef. Previous reports indicate that an alanine instead of a leucine residue at position Y+3 severly impairs interaction between tyrosine-based motifs and μ subunits (Boll et al., 1996; Ohno et al., 1996). A likely hypothesis to account for the effect of Nef is that direct or indirect interactions of MHC I with AP complexes are mediated by the viral protein. Our colocalization experiments are consistent with this hypothesis. In the absence of Nef, HLA-A and -B molecules are found largely at the cell surface and colocalize poorly with AP complexes, whereas in the presence of Nef they accumulate in a region containing clathrin-coated vesicles budding from the Golgi that contain AP-1. This does not exclude a stimulation of the endocytosis of cell surface molecules by Nef. Both mechanisms have been shown to participate in the modulation of CD4 induced by Nef (Greenberg et al., 1997; Mangasarian et al., 1997).

The action of Nef on the cell-sorting machinery was evidenced by yeast two-hybrid and cell-free assays, showing binding of Nef proteins from HIV-1, HIV-2, and SIV to μ1 and μ2 subunits of AP complexes and not to α, β, or γ adaptins. HIV-1Nef bound weakly to μ1 but not detectably to μ2 in the yeast assay, whereas the viral protein recognized both μ1 and μ2 in a cell-free assay. A specific interaction between complete AP-1 complexes from HeLa-CD4 lysates and HIV-1Nef was also detected. Analysis of deletion mutants indicated that a region (aa 143–170) corresponding to a disordered loop in the crystal structure of HIV-1Nef (Lee et al., 1996) is involved in the interaction with μ1. Nef proteins from HIV-2 and SIV interacted strongly with μ1 and μ2. Although caution should be taken in extrapolating these results to in vivo models, it is presumable that HIV-1Nef acts preferentially on the AP-1 sorting pathway. Interaction of Nef with μ2 indicates that the internalization of surface MHC I molecules that have escaped the incorporation into clathrin-coated vesicles at the TGN may also be stimulated by the viral protein.

The specific recruitment of HLA-A and -B by the sorting machinery in the presence of Nef may operate in different ways (Figure 7, model). Conformational or post-translational changes of the μ chains induced by Nef may facilitate the recognition of the tyrosine-based motif (Figure 7B). The μ1 and μ2 subunits as well as α, β1, and β2 chains are phosphorylated in vivo. Phosphorylation influences adaptor–clathrin interactions and could play a role in controlling coat assembly and disassembly (Wilde and Brodsky, 1996). Nef may affect the phosphorylation of APs and subsequently the recognition of HLA-A and -B cytoplasmic domains. Alternatively, Nef could serve as a connector between AP and HLA-A and -B molecules in a way similar to that proposed for the modulation of CD4 (Mangasarian et al., 1997). Our failure to demonstrate direct binding of Nef to HLA-A and -B by coimmunoprecipitation and by yeast two-hybrid assays (data not shown) may reflect the low stability of Nef-MHC I complexes. Interaction between CD4 and Nef has not been conclusively demonstrated in mammalian

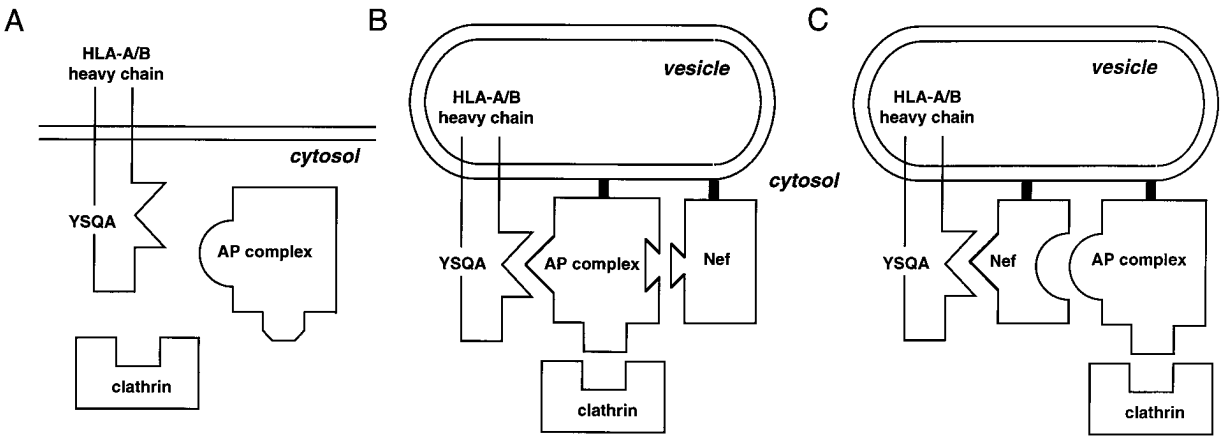

Figure 7. Models for Nef-Induced MHC I Modulation

(A) In the absence of Nef, the tyrosine-based motif YSQA of HLA-A and -B cytoplasmic domains is not recognized by the medium chains of the AP-1 or AP-2 complexes. MHC I molecules are addressed to the cell surface where they are stably expressed.

(B and C) Models for HLA-A and -B modulation induced by Nef. Binding of Nef to medium chains (μ1 or μ2) leads to the association of AP complexes with HLA-A and -B heavy chains. Nef may act by inducing post-translational or conformational modifications of μ subunits, allowing the recognition of the YSQA motif by AP complexes (B) or as a connector between the MHC I cytoplasmic domain and μ subunits (C). Clathrin-coated vesicles are subsequently formed at the TGN (association with AP-1 complexes) or at the plasma membrane (association with AP-2) leading to the intracellular accumulation of MHC I molecules.

cells, although binding was observed in cell-free assays, in insect cells, and in the yeast two-hybrid system (Harris, 1996). It is likely that similar mechanisms are used by Nef to modulate MHC I and CD4, since both molecules are affected at the same steps of their transport pathway. Furthermore, dileucine and tyrosine-based motifs mediate addressing to similar subcellular compartments, and it has been reported that a dileucine motif can also be recognized by AP-1 and AP-2 complexes in cell-free assays (Heilker et al., 1996).

Numerous pathogenic viruses escape detection by the immune system by down-regulating MHC I surface expression. Nef-induced MHC I modulation protects infected cells against lysis by specific cytotoxic T lymphocytes (Collins et al., 1998). However, modulating MHC I renders target cells more sensitive to NK lysis (Brutkiewicz and Welsh, 1995). HLA-C molecules, in contrast with HLA-A and -B, are dominant inhibitory ligands protecting against NK lysis (Colonna et al., 1993) and are not affected by Nef. One can speculate that, due to Nef expression, in vivo infected cells display limited recognition by cytotoxic T lymphocytes without increased lysis by NK cells. Escaping the immune surveillance by both cytotoxic T lymphocytes and NK may contribute significantly to the pathogenesis of HIV.

Experimental Procedures

Plasmid Construction

MHC I Expression Vectors

The HLA-A2, -B7, and Cw3 vectors were a kind gift from F. Lemonnier (Institut Pasteur, Paris, France). The *HLA-A2* gene was subcloned in a BS plasmid (Biolabs), yielding pBA2. The *HLA-A2* gene has unique sites at the introns located between exons 5 and 6 (AflII), exons 6 and 7 (NcoI), and exons 7 and 8 (SphI). HLA-A2 mutants lacking either exon 6 or 7 (HLA-A2Δ6 and HLA-A2Δ7, respectively) were constructed by using these sites. HLA-A2 WT and mutant genes were subcloned downstream of the CMV promoter in pcDNA3 (Invitrogen), yielding A2 WT, A2Δ6, and A2Δ7 vectors, respectively. Immunoprecipitation experiments indicated that A2Δ6 and A2Δ7 have an apparent molecular weight consistent with the deletions (data not shown). Point mutants of HLA-A2 exon 6 were constructed by polymerase chain reaction (PCR). Subcloning in pcDNA3 yielded A2 Y320A, A2 Y320F, and A2 SQ321AL vectors. The coding region for the cytoplasmic tail of HLA-Cw3 was amplified by PCR. The corresponding HLA-A2 fragment was then replaced by Cw3 in A2 WT, yielding A2-CW3 vector. The sequence of *HLA-A2* derivatives was verified by sequencing. The aa numeration is according to Vega and Strominger (1989).

Nef Expression Vectors

The Nef-FT vector carrying the *nef LAI* gene is a gift from F. Bachelerie (Institut Pasteur) (Bachelerie et al., 1990). The *nef* gene from the HIV-1 primary strain A01 (Schwartz et al., 1993) was cloned in the same CMV-driven vector, yielding Nef A01. The control Nef-mock plasmid carries the *nef* A01 gene in an antisense orientation. CMVNef-HIV-2ST and CMVNefSIVmac239 plasmids were provided by C. Aiken (Salk Institute, La Jolla, California). The Nef vectors used in two-hybrid assays were constructed as described for the NefHIV-1Lai vector (Benichou et al., 1994). The HIV-2Rod and SIVmac239 *nef* genes were amplified by PCR and inserted in frame with Gal4BD in the pGBT10 plasmid. The N- and C-terminal deletion mutants (NefΔ1–NefΔ6) of NefLai were constructed by PCR and subcloning in pGBT10. The GST-NefSIVmac vector was constructed as described for GST-NefHIV-1Lai (Bodeus et al., 1995) in the pGEX-4T1 plasmid (Pharmacia).

CD25 Vector

The cDNA of the α chain of the IL-2 receptor cloned in a pCDM8 vector (CD25 vector) is a gift from A. Dautry-Varsat (Institut Pasteur).

μ1, μ2, and Adaptins Vectors

Plasmids for expression of μ1 and μ2, α, γ, β1, and β2 subunits of AP-1 and AP-2 complexes fused to the Gal4AD in the pACTII vector were provided by J. S. Bonifacino (National Institutes of Health, Bethesda, Maryland) (Ohno et al., 1995). For in vitro translation, the μ1 and μ2 open reading frames were subcloned downstream of the T7 promoter, in the pSG-Flag plasmid (a gift from P. Jalinot, Lyon, France).

Cells, Virus, and Antibodies

HeLa and HeLa-CD4 cells were grown and HIV-1 (NL43 strain) was produced as described (Schwartz et al., 1995b). The following antibodies were used: anti-CD4 mAb: Leu3a-phycoerythrin (PE), Becton Dickinson; anti-CD25: 33B3-fluorescein isothiocyanate, Immunotech, and anti-IL2R-PE, Becton Dickinson; and anti-HLA-A, -B, -C

heavy chain: W6/32-fluorescein isothiocyanate, Sera-Lab. Anti-HLA-A2 (BB7.2) and anti-HLA-B7 (BB7.1) mAbs provided by F. Lemonnier (Institut Pasteur) (Ferrier et al., 1985; Schwartz et al., 1996). The anti-Nef mAb MATG 020 is a gift from Transgene (Strasbourg, France). The mAb L31 specific for HLA-C free heavy chains (Ciccone et al., 1995) is a gift from A. Beretta (Hopital St Joseph, Paris, France). Rabbit anti-clathrin antibody was provided by M. Robinson (University of Cambridge, Cambridge, UK) (Robinson, 1987, 1993) and anti-Rab6 antibody by B. Goud (Institut Curie, Paris, France) (Goud et al., 1990). Anti-γ (clone 100/3) and anti-$\beta1+\beta2$ adaptins mAb were from Sigma and Transduction, respectively. Secondary antibodies were from Southern Biotech and Amersham.

Transfection, HIV-1 Infection, Flow Cytometry, and Western Blot Analysis

Electroporation, HIV-1 infection, Western blot, and flow cytometry analysis were performed as described (Schwartz et al., 1995b, 1996). Staining with the L31 mAb that selectively binds to HLA-C free chains was performed as described (Ciccone et al., 1995), by treating cells in an acidic buffer (10% fetal calf serum-RPMI acidified at pH 2.2 by addition of 2 N HCl) to remove β_2-microglobulin, thus allowing exposure of HLA-C epitopes to L31.

Indirect Immunofluorescence Stainings

After electroporation, 2×10^5 HeLa cells were spread on glass coverslips in 24-well plates and stained for immunofluorescence 24–40 hr later. For MHC I, HLA-A2, Nef, Rab6, clathrin, and mouse anti-γ adaptin stainings, cells were fixed in 3.7% paraformaldehyde–phosphate-buffered saline. For anti-clathrin stainings (Robinson, 1987, 1993) cells were fixed and permeabilized in methanol for 5 min at -20°C followed by air drying. Cells were stained and analyzed as described (Schwartz et al., 1996). Confocal microscopy was performed on a Leica TCS4D instrument. Series of optical sections at 0.3–0.7 μm intervals were recorded and mounted using the Adobe Photoshop software.

Two-Hybrid Assay

The HF7c and SFY526 yeast reporter strains containing the HIS3 and LacZ Gal4-inducible genes, respectively, were cotransformed as described (Benichou et al., 1994) and plated on selective medium lacking tryptophan and leucine. HF7c double transformants were patched on the same media and analyzed for histidine auxotrophy by replica-plating on selective medium lacking tryptophan, leucine, and histidine. 5 mM 3-amino-1,2,4-aminotriazole was added to abrogate transcriptional background observed with the NefHIV-2Rod and NefSIVmac239 hybrids. SFY526 double transformants were patched on selective medium and analyzed for β-gal expression (Benichou et al., 1994).

Cell-Free Binding Assay

Binding of Nef to μ subunits

Bacterially expressed GST-NefHIV-1Lai and GST-NefSIVmac239 proteins were purified and immobilized on GSH-agarose beads (Benichou et al., 1994). [^{35}S] μ1 and μ2 proteins were prepared from the pSGFlag-μ1 and -μ2 plasmids using the TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]methionine. [^{35}S]-μ1 or -μ2 were incubated overnight at 4°C with 2 μg of GST-NefHIV-1Lai, GST-NefSIVmac239, or GST immobilized on GSH-agarose beads in phosphate-buffered saline containing 2 mg/ml bovine serum albumin and 0.05% Tween. Beads were washed three times with 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 300 mM NaCl, 10% glycerol, and 1% Nonidet P-40. Bound labeled proteins were resolved by SDS-PAGE and revealed by autoradiography.

Binding of Nef to cellular AP-1 complexes

The 25×10^6 HeLa-CD4 cells were lysed in 50 mM Tris-HCl [pH 8], 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100. Lysates were incubated overnight at 4°C with 2 μg of GST-NefHIV-1Lai or GST immobilized on GSH-agarose beads. Beads were washed five times in lysis buffer. Bound cellular proteins were analyzed by Western blotting using anti-γ and anti-$\beta1+\beta2$ adaptin mAbs.

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References

Aiken, C., and Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J. Virol. *69*, 5048–5056.

Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E., and Trono, D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell *76*, 853–864.

Bachelerie, F., Alcami, J., Hazan, U., Israël, N., Goud, B., Arenzana-Seisdedos, F., and Virelizier, J.L. (1990). Constitutive expression of human immunodeficiency virus (HIV) nef protein in human astrocytes does not influence basal or induced HIV long terminal repeat activity. J. Virol. *64*, 3059–3062.

Benichou, S., Bomsel, M., Bodeus, M., Durand, H., Doute, M., Letourneur, F., Camonis, J., and Benarous, R. (1994). Physical interaction of the HIV-1 Nef protein with beta-COP, a component of non-clathrin-coated vesicle for membrane traffic. J. Biol. Chem. *269*, 30073–30076.

Benmerah, A., Bègue, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996). The ear of α-adaptin interacts with the COOH-terminal domain of the Eps15 protein. J. Biol. Chem. *271*, 12111–12116.

Bijlmakers, M.J., and Ploegh, H.L. (1993). Putting together an MHC class I molecule. Curr. Opin. Immunol. *5*, 21–26.

Bodeus, M., Marie-Cardine, A., Bougeret, C., Ramos-Morales, F., and Benarous, R. (1995). In vitro binding and phosphorylation of human immunodeficiency virus type 1 Nef protein by serine/threonine protein kinase. J. Gen. Virol. *76*, 1337–1344.

Boll, W., Ohno, H., Songyang, Z., Rapoport, I., Cantley, L.C., Bonifacino, J.S., and Kirchhausen, T. (1996). Sequence requirements for the recognition of tyrosine-based endocytic signals by clathrin AP-2 complexes. EMBO J. *15*, 5789–5795.

Brutkiewicz, R.R., and Welsh, R.M. (1995). Major histocompatibility complex class I antigens and the control of viral infections by natural killer cells. J. Virol. *69*, 3967–3971.

Ciccone, E., Pende, D., Nanni, L., Di Donato, C., Viale, O., Beretta, A., Vitale, M., Sivori, S., Moretta, A., and Moretta, L. (1995). General role of HLA class I molecules in the protection of target cells from lysis by natural killer cells: evidence that the free heavy chains of class I molecules are not sufficient to mediate the protective effect. Int. Immunol. *7*, 393–400.

Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature *391*, 397–401.

Colonna, M., Borsellino, G., Falco, M., Ferrara, G.B., and Strominger, J.L. (1993). HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. Proc. Natl. Acad. Sci. USA *90*, 12000–12004.

Ferrier, P., Layet, C., Caillol, D.H., Jordan, B.R., and Lemonnier, F.A. (1985). The association between murine β2-microglobulin and HLA class I heavy chains results in serologically detectable conformational changes of both chains. J. Immunol. *135*, 1281–1287.

Foti, M., Mangasarian, A., Piguet, V., Lew, D.P., Krause, K.H., Trono, D., and Carpentier, J.L. (1997). Nef-mediated clathrin-coated pit formation. J. Cell Biol. *139*, 37–47.

Garcia, J.V., and Miller, A.D. (1991). Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature *350*, 508–511.

Goud, B., Zahraoui, A., Tavitian, A., and Saraste, J. (1990). Small GTP-binding proteins associated with Golgi cisternae. Nature *345*, 553–556.

Greenberg, M.E., Bronson, S., Neumann, M., Pavlakis, G.N., and Skowronski, J. (1997). Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. *16*, 6964–6976.

Guild, B., and Strominger, J.L. (1984). Human and murine class I MHC antigens share conserved serine 335, the site of HLA phosphorylation in vivo. J. Biol. Chem. *259*, 9235–9240.

Harris, M. (1996). From negative factor to a critical role in virus pathogenesis: the changing fortune of Nef. J. Gen. Virol. *77*, 2379–2392.

Heilker, R., Manning-Krieg, U., Zuber, J.F., and Spiess, M. (1996). In vitro binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting. EMBO J. *15*, 2893–2899.

Kestler, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D., and Desrosiers, R.C. (1991). Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. Cell *65*, 651–662.

Lee, C.H., Saksela, K., Mirza, U.A., Chait, B.T., and Kuriyan, J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell *85*, 931–942.

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

Marks, M.S., Ohno, H., Kirchhausen, T., and Bonifacino, J.S. (1997). Protein sorting by tyrosine-based signals: adapting to the Ys and wherefores. Trends Cell Biol. *7*, 124–128.

Neefjes, J.J., Stollorz, V., Peters, P.J., Geuze, H.J., and Ploegh, H.L. (1990). The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. Cell *61*, 171–183.

Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I., Miyakate, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J.S. (1995). Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science *269*, 1872–1875.

Ohno, H., Fournier, M.C., Poy, G., and Bonifacino, J.S. (1996). Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J. Biol. Chem. *271*, 29009–29015.

Parham, P., Adams, E.J., and Arnett, K.L. (1995). The origins of HLA-A,B,C polymorphism. Immunol. Rev. *143*, 141–180.

Pelchen-Mattews, A., Parsons, I.J., and Marsh, M. (1993). Phorbol ester-induced downregulation of CD4 is a multistep process involving dissociation from p56^{lck}, increased association with clathrin-coated pits, and altered endosomal sorting. J. Exp. Med. *178*, 1209–1222.

Reid, P.A., and Watts, C. (1990). Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. Nature *346*, 655–657.

Robinson, M.S. (1987). 100-kD coated vesicle proteins: molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. J. Cell Biol. *104*, 887–95.

Robinson, M.S. (1993). Assembly and targeting of adaptin chimeras in transfected cells. J. Cell Biol. *123*, 67–77.

Robinson, M.S. (1994). The role of clathrin, adaptors and dynamin in endocytosis. Curr. Opin. Cell Biol. *6*, 538–544.

Sandoval, I.V., and Bakke, O. (1994). Targeting of membrane proteins to endosomes and lysosomes. Trends Cell Biol. *4*, 292–297.

Schwartz, O., Rivière, Y., Heard, J.M., and Danos, O. (1993). Reduced cell surface expression of processed HIV-1 envelope glycoprotein in the presence of Nef. J. Virol. *67*, 3274–3280.

Schwartz, O., Dautry-Varsat, A., Goud, B., Maréchal, V., Subtil, A., Heard, J.M., and Danos, O. (1995a). HIV-1 Nef induces accumulation of CD4 in early endosomes. J. Virol. *69*, 528–533.

Schwartz, O., Maréchal, V., Danos, O., and Heard, J.M. (1995b). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. J. Virol. *69*, 4053–4059.

Schwartz, O., Maréchal, V., Le Gall, S., Lemonnier, F., and Heard, J.M. (1996). Endocytosis of MHC I molecules is induced by HIV-1 Nef. Nat. Med. *2*, 338–342.

Stang, E., Kartenbeck, J., and Parton, R.G. (1997). Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. Mol. Biol. Cell *8*, 47–57.

Trowbridge, I.S., Collawn, J.F., and Hopkins, C.R. (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. *9*, 129–161.

Vega, M.A., and Strominger, J.L. (1989). Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. Proc. Natl. Acad. Sci. USA *86*, 2688–2692.

Wilde, A., and Brodsky, F.M. (1996). In vivo phosphorylation of adaptors regulates their interaction with clathrin. J. Cell Biol. *135*, 635–645.