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# Leucine-Specific, Functional Interactions between Human Immunodeficiency Virus Type 1 Nef and Adaptor Protein Complexes

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**The human immunodeficiency virus type 1 virulence protein Nef interacts with the endosomal sorting machinery via a leucine-based motif. Similar sequences within the cytoplasmic domains of cellular transmembrane proteins bind to the adaptor protein (AP) complexes of coated vesicles to modulate protein traffic, but the molecular basis of the interactions between these motifs and the heterotetrameric complexes is controversial. To identify the target of the Nef leucine motif, the native sequence was replaced with either leucine- or tyrosine-based AP-binding sequences from cellular proteins, and the interactions with AP subunits were correlated with function. Tyrosine motifs predictably modulated the interactions between Nef and the μ subunits of AP-1, AP-2, and AP-3; heterologous leucine motifs caused little change in these interactions. Conversely, leucine motifs mediated a ternary interaction between Nef and hemicomplexes containing the σ1 plus γ subunits of AP-1 or the σ3 plus δ subunits of AP-3, whereas tyrosine motifs did not. Similarly, only leucine motifs supported the Nef-mediated association of AP-1 and AP-3 with endosomal membranes in cells treated with brefeldin A. Functionally, Nef proteins containing leucine motifs down-regulated CD4 from the cell surface and enhanced viral replication, whereas those containing tyrosine motifs were inactive. Apparently, the interaction of Nef with the μ subunits of AP complexes is insufficient for function. A leucine-specific mode of interaction that likely involves AP hemicomplexes is further required for Nef activity. The μ and hemicomplex interactions may cooperate to yield high avidity binding of AP complexes to Nef. This binding likely underlies the unusual ability of Nef to induce the stabilization of these complexes on endosomal membranes, an activity that correlates with enhancement of viral replication.**

The Nef protein of human immunodeficiency virus type 1 (HIV-1) is required for efficient pathogenesis (10, 26). This peripheral membrane protein alters the intracellular trafficking of various transmembrane proteins. For example, Nef increases the endocytosis, lysosomal targeting, and degradation of CD4, the primary receptor for HIV-1 (1), and it retains major histocompatibility complex (MHC) class I in the *trans*-Golgi region (18, 49). Nef causes the assembly of maximally infectious virions, and it increases the replication rate of HIV-1 in vitro (6, 35, 50). The basis of the virologic effects of Nef may be multifactorial, reflecting both the enhancement of particle infectivity, due in part to relief of an inhibitory effect of CD4 on the incorporation of the envelope glycoprotein into the virion (27), and an optimization of the transcriptional environment caused by Nef-mediated facilitation of T-cell activation (32, 52). Nevertheless, several of these properties (down-regulation of cell surface CD4, enhancement of viral infectivity, and maximal viral growth rate in vitro) require a leucine-based protein sorting motif located within a 30-residue, solvent-exposed, unstructured loop near the C terminus of the Nef protein (7). This leucine-based sorting motif is conserved throughout the primate lentiviruses and conforms to the sequence E/DXXXLϕ, where ϕ is a residue with a hydrophobic side chain.

The leucine-based motif in Nef is homologous to those found within the cytoplasmic domains of several mammalian and yeast proteins (9, 22, 44). These motifs, like the tyrosine-based motifs of the sequence YXXϕ, interact with the adaptor protein (AP) complexes. The AP complexes form part of the cytoplasmic coat of vesicles involved in post-Golgi protein transport, where they mediate the selective inclusion of transmembrane proteins into the forming vesicles (reviewed in references 20 and 28). There are four members of the AP complex family, and each member consists of four distinct subunits. The recognition of specific sorting signals by the distinct types of AP complexes presumably contributes to the specificity of transport between post-Golgi membranous structures such as the *trans*-Golgi network (TGN), endosomes, lysosomes, and the plasma membrane.

Specific sorting signals bind preferentially to distinct AP complexes and/or their subunits. For example, the mammalian lysosomal integral membrane protein LIMP II and the melanosomal protein tyrosinase contain sequences that conform to the consensus E/DXXXLϕ; these proteins are targeted to ly-

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sosomes and lysosome-like organelles via vesicles coated with AP-3 (22). In contrast, the E/DXXXLϕ sequence within the T-cell receptor (TCR) γ chain, although able to mediate lysosomal targeting (30), appears to direct interactions with AP-1, an adaptor complex associated with vesicular transport between the TGN and the endosomal-lysosomal system, and AP-2, the adaptor complex associated with endocytic vesicles (12, 30). Although the sequence requirements for the recognition of specific AP complexes by leucine-based motifs are unclear, the acidic residue(s) at position −4 and/or −5 relative to the leucines appears necessary for the interaction with AP-3 both in vitro and in vivo (9, 22). The tyrosine-based motifs also mediate specific AP-binding preferences. For example, the tyrosine-based motif in the lysosomal protein LAMP I (lgp120) binds preferentially to the medium (μ) subunit of AP-3 (51), whereas the TGN resident protein TGN38 binds with similar avidities to the μ subunits of AP-1, -2, and -3 (11, 38, 39).

Although the leucine- and tyrosine-based motifs have similar functions as sorting signals, they may recognize distinct sites on the AP complex; this hypothesis is supported by the observation that protein trafficking mediated by these two types of motifs is independently saturable (34). Furthermore, structural data describing the binding site on μ subunits for the tyrosine motifs suggest that this site is unlikely to accommodate acidic, leucine-based sequences (40). Although virtually all of the available evidence indicates that the tyrosine-based motifs interact with the μ subunits of AP complexes (37–40), the molecular partner of leucine-based motifs is controversial. Chemical cross-linking studies have suggested an interaction between the leucine motif of the TCR γ chain and the large β subunits of AP-1 and AP-2 (17, 46), whereas filter-binding and surface plasmon resonance assays have indicated an interaction between the acidic leucine motif of the invariant chain and the μ subunits of these AP complexes (21, 48).

The molecular basis of the interaction between Nef and the AP complexes is also controversial. HIV-1 Nef interacts with the μ subunits of AP complexes (29, 42). Quantitative yeast two-hybrid assays indicate that the interactions with μ1 and μ3 are stronger than the interaction with μ2 (8). Although the Nef-μ3 and Nef-μ2 interactions appear to require the leucine-based motif, the Nef-μ1 interaction is attenuated but not abolished by mutation of the leucines (8). In addition to the leucine-based motif, sequences within the structured regions of Nef that flank the C-terminal loop also contribute to the μ interaction (14). Circumstantial evidence indicates that Nef interacts with the β subunits of AP-1 and/or AP-2: a peptide containing the leucine motif of Nef competes with a peptide containing the leucine motif of the TCR γ chain for binding in vitro to β1 and β2 (17). In addition, Nef reportedly interacts in a leucine-dependent fashion with β1 and β2 subunits in yeast two-hybrid assays (16). Recently, yeast three-hybrid assays have revealed a leucine-dependent ternary interaction between Nef and AP hemicomplexes containing two of the four subunits: the σ1 plus γ subunits of AP-1 or the σ3 plus δ subunits of AP-3 (25). No interactions were observed between Nef and the analogous hemicomplexes of AP-2 or AP-4 or between Nef and any μ plus β subunit hemicomplex.

Although the roles of the specific subunits are uncertain, the leucine-based motif in Nef is clearly required for the interaction with intact AP-1 and AP-3 in vitro (3, 24). Furthermore, leucine-dependent binding correlates with the unusual ability of Nef to stabilize the membrane association of AP-1 and AP-3 in vivo by a mechanism independent of ARF1, the cellular GTP-binding protein required for their attachment under physiologic conditions (24). These findings suggest that Nef interacts with an unusually high affinity with these complexes, but they leave open the specific role of the leucine-based motif.

Is a leucine-based motif specifically required for complex stabilization and Nef function, or would any AP-binding motif be sufficient? Does the leucine-based motif in Nef have unique features that confer an unusual interaction with AP complexes? What can the study of Nef reveal about the general function of leucine-based motifs? To answer these questions, we replaced the native leucine motif in Nef with a variety of leucine and tyrosine motifs found in cellular proteins and correlated protein interactions with biologic activity. Functional data indicate that the activity of Nef can be supported by a variety of leucine-based sorting motifs but not by tyrosine-based motifs. Binding data suggest that leucine-based motifs interact with the complexes via a mechanism distinct from that of tyrosine-based motifs, and this distinct mode of interaction is required for Nef function. We present the hypothesis that Nef binds AP complexes via two interactions: sequences flanking the loop bind the μ subunits, while the leucine-based motif within the loop binds the σ plus large, specific subunit hemicomplex. Together, these interactions yield the unusually high affinity for AP complexes required for Nef function.

## MATERIALS AND METHODS

**DNA constructions.** For mutagenesis of HIV-1 *nef*, overlap PCR was used to create substitutions of the native amino acid sequence GENTSLL with heterologous sorting motifs derived from cellular proteins (TCR γ, GDKQTLL; Limp II, DERAPLI; tyrosinase, EERQPLL; TGN38, GSDYQRL; lgp120, GAGYQT I). The mutated *nef* sequences were inserted into the mammalian expression vector pCIneo (Promega) by using the EcoRI and SalI sites and were confirmed by nucleotide sequence analysis. To construct transmembrane chimeras with Nef as the cytoplasmic domain, mutated *nef* sequences were transferred into CD8-Nef (14) by using the CelII and BspE1 sites within *nef*. To construct proviral mutants, *nef* sequences were transferred into pNL4-3 by using the XhoI and PmlI sites within *nef*. The mutated sequences were also transferred into the yeast two-hybrid vector pGBKT7 (Clontech) by using the EcoRI and SalI sites to place the entire *nef* sequence in frame with the Gal4 DNA-binding domain. For yeast three-hybrid analyses, pBridge-based vectors encoding Nef as a fusion with the Gal4 DNA-binding domain and encoding the σ subunits of AP-1 or -3 were provided by Juan Bonifacino (25); the mutant *nef* sequences were inserted into the pBridge-derived vectors by using the BspEI-BlpI sites within Nef for the σ1 vector and the EcoRI and SalI sites for the σ3 vector. The yeast expression vectors encoding the mannose 6-phosphate receptor cytoplasmic domain, the μ subunits (μ1, μ2, and μ3), and the GGA proteins were provided by Juan Bonifacino (38, 45). The yeast expression vector encoding the β1 subunit was provided by Margaret Robinson (41). The yeast expression vector encoding the H subunit of vATPase (residues 133 to 483) was provided by Matija Peterlin (31). The yeast expression vector encoding β-COP was described previously (2). pGADT7-derived yeast expression vectors encoding the γ and δ subunits were provided by Juan Bonifacino (25). The empty vector controls pACT2 and pBridge were obtained from Clontech. The pCIneo derivative expressing wild-type Nef from pNL4-3 (pCINL), the pNL4-3 derivative in which the *nef* gene is disrupted by two premature termination codons (pDS), and the pNL4-3 derivative that contains alanine substitutions of the Nef dileucine motif were described previously (6, 7).

**Cells and transfections.** For assays of Nef-mediated down-regulation of CD4 and MHC class I, HEK 293 cells were maintained in minimal essential medium with Earle's balanced salts plus 10% fetal bovine serum (FBS), and the cells were transfected with pCIneo-based Nef expression vectors, a green fluorescent protein (GFP) expression vector (phGFP-S65T; Clontech), and for the CD4 assays, a CD4 expression vector (pCMX-CD4) by calcium phosphate-DNA coprecipi-

TABLE 1. AP-binding motifs

| Sequence | Previously described characteristics[a] |
|---|---|
| GENTSLL | Wild-type sequence of $Nef_{NL4-3}$, the LL is required for Nef-mediated down-regulation of CD4 and optimal viral infectivity |
| EERQPLL | From the melanosomal protein tyrosinase, binds selectively to intact AP-3 in vitro, EE and LL sequences required for binding |
| GDKQTLL | From the TCR γ chain, binds intact AP-1 and AP-2 in vitro, causes lysosomal targeting |
| DERAPLI | From LIMP II, binds intact AP-1 and AP-3 in vitro, DE sequence required for binding to AP-3 |
| GAGYQTI | From the lysosomal glycoprotein 120 (LAMP I), binds preferentially to the μ subunit of AP-3 in yeast two-hybrid assays |
| GSDYQRL | From the TGN38 protein, binds to the μ subunits of AP-1, -2, and -3 in yeast two-hybrid assays |

[a] Referenced in the text.

tation with the reagents and procedures of the CellPhect transfection kit (Pharmacia) or by complexing the DNA with cationic liposomes (Lipofectamine 2000). CD4-positive HeLa cells of clone 1022 were maintained in G418 (5) and were transfected for flow cytometry with Lipofectamine 2000 or for immunofluorescence (IF) microscopy by using Lipofectin according to the manufacturer's instructions (BRL). T cells of the continuous human line CEM were maintained in RPMI 1640 plus 10% FBS and were cocultured with transiently transfected 293 cells to produce viral stocks as described below. CD4-positive HeLa cells (clone P4.R5) were maintained in Dulbecco's minimal essential medium plus 10% FBS and puromycin and were used for viral infectivity assays. Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-seronegative volunteers and incubated in RPMI 1640 with 10% FBS until activation with phytohemagglutinin (3 μg/ml) and interleukin-2 (20 U/ml) before infection during the viral growth rate assays.

**Flow cytometry.** For assays of Nef-mediated CD4 down-regulation, transfected 293 cells were stained with phycoerythrin (PE)-conjugated anti-CD4 (OKT4; Becton Dickinson), fixed in paraformaldehyde, gated by forward- and side-scatter characteristics, and then analyzed by two-color flow cytometry for GFP and PE fluorescence. Assays of down-regulation of class I were performed similarly to those for CD4, except that PE-conjugated anti-class I A, B, and C heavy chain (Pharmingen) was used to detect endogenous class I on the 293 cells. For assay of the expression of CD8-Nef chimeras at the cell surface, HeLa 1022 cells were transfected with Lipofectamine 2000, stained with anti-CD8-fluorescein isothiocyanate (FITC) or an FITC-conjugated antibody isotype control (Becton Dickinson), fixed in paraformaldehyde, gated by forward- and side-scatter characteristics, and analyzed for FITC fluorescence.

**Yeast two-hybrid and three-hybrid assays.** For the two-hybrid assays, yeast cells (*Saccharomyces cerevisiae* strain HF7c) were cotransformed with pGBT9- or pGBKT7- and pACT2-based plasmids by a lithium acetate procedure as described in the Matchmaker kit (Clontech) and then plated on leucine- and tryptophan-minus (2-minus) media. From each plate, 5 to 8 cotransformed colonies were pooled and then grown overnight in 2-minus media. For quantitative growth rate assays, a 0.005 optical density at 600 nm of yeast from the overnight cultures were used to inoculate 5 ml of leucine-, tryptophan-, and histidine-minus media, and the optical density at 600 nm was measured spectrophotometrically during incubation at 30°C. For the three-hybrid assays, cells were cotransformed with pBridge- and pGADT7-based plasmids and then plated on leucine- and tryptophan-minus media. For patching, 15 colonies were plated in duplicate on leucine- and tryptophan-minus or leucine-, tryptophan-, and methionine-minus media. After incubation for 1 to 2 days, the patches were replica plated onto media with and without histidine and incubated at 30°C.

**Viral infectivity and growth rate assays.** To produce virus from CD4-negative cells, 293 cells ($2 \times 10^6$ in each 10-cm-diameter dish) were transfected with pNL4-3 or its *nef* mutant derivatives by using 10 μg of plasmid DNA and Lipofectamine 2000. After 2 days, the supernatants were clarified by centrifugation at $800 \times g$ and filtration through 0.22-μm-pore-size membranes. To produce virus from CD4-postitive cells, 2 days after transfection of 293 cells ($5 \times 10^5$ in each well of a six-well plate with 2 μg of DNA), the supernatants were removed and replaced with 3 ml of media (RPMI 1640 plus 10% FBS) containing $3 \times 10^6$ CEM cells. After 2 days of cocultivation, the supernatants were collected and clarified by centrifugation at $800 \times g$. The concentration of p24 antigen was determined for each viral stock by enzyme-linked immunosorbent assay (Coulter). Viral infectivity was determined as previously described. Serial dilutions of each viral stock were used to infect HeLa-CD4 cells (clone P4.R5) in duplicate; these cells contain a long terminal repeat-driven β-galactosidase indicator construct. After 2 days, the cells were fixed with 1% formaldehyde–0.2% glutaraldehyde in phosphate-buffered saline and then stained with 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal), and the numbers of infectious centers observed microscopically were counted. The ratio of infectious centers per picogram of p24 antigen for each viral stock was normalized to a wild-type value of 100 (percent infectivity). For the growth rate assays, 30 ng of p24 antigen from each viral stock derived from the 293/CEM cocultures was used to inoculate $10^6$ PBMC blasts prepared as described above. After an overnight incubation, the cells were washed and then incubated in media plus interleukin-2. The concentration of p24 in the culture supernatants was measured by enzyme-linked immunosorbent assay.

**Immunofluorescence microscopy.** HeLa-CD4 cells were transfected as described above, fixed, permeabilized, and stained as described previously (8). CD8-Nef chimeras were detected by direct IF; the γ subunit of AP1 and the δ subunit of AP-1 were detected by indirect IF, as described previously (24). Images were acquired by using a Zeiss microscope with a Bio-Rad laser scanning confocal attachment by using single-laser excitation and were processed by using Adobe Photoshop software.

**Glutathione *S*-transferase (GST) pull-down assays.** Pull-down assays were performed as described previously (24) by using fusion proteins purified from *Escherichia coli*, glutathione-agarose beads, and cytoplasmic lysates from HeLa cells.

## RESULTS

**Experimental strategy.** Mutational analyses designed to associate protein interactions with biologic activity usually depend on loss-of-function correlations. Here, we used a substitution strategy in which characterized AP-binding motifs were inserted in place of the native sequence within the C-terminal loop of Nef. The evaluation of tyrosine-based sequences known to bind μ subunits tested the hypothesis that the μ interaction is sufficient for Nef function, whereas the evaluation of leucine-based sequences known to bind AP complexes allowed a rigorous test of the roles of the μ, β, or AP hemicomplex interactions. The following AP-binding motifs listed in Table 1 were evaluated by inserting them in place of the Nef sequence ENTSLL, taking care not to change the 30-residue length of the loop (see Materials and Methods): the tyrosine-based sequence SDYQRL from the *trans*-Golgi-associated protein TGN38, which interacts with μ1, μ2, and μ3 in yeast two-hybrid assays; the sequence AGYQTI from the lysosomal glycoprotein lgp120 (LAMP I), which binds preferentially to μ3 (11, 38, 39, 51); the leucine-based sequence DERAPLI from the lysosomal integral membrane protein LIMP II and the sequence EERQPLL from the melanosomal protein tyrosinase, both of which interact preferentially with intact AP-3 (and AP-1 in the case of LIMP II) by surface plasmon resonance (15, 22); and the sequence DKQTLL from the TCR γ

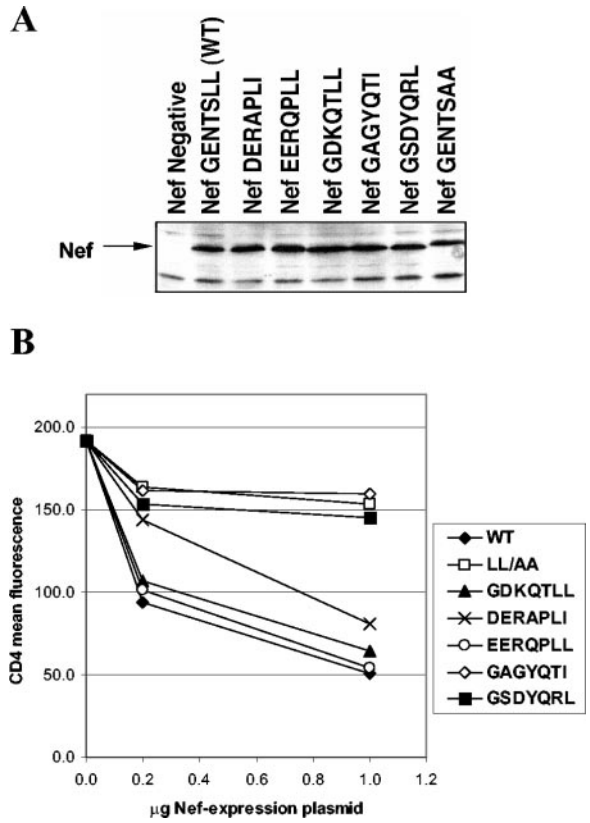


FIG. 1. Expression and down-regulation of surface CD4 by mutated Nef proteins. (A) Expression. Cells (HEK 293) were transfected with full-length HIV-1 proviral plasmids (pNL4-3 based) encoding the indicated Nef protein sequences. The Western blot was performed as described previously (7). The arrow indicates the position of 27-kDa Nef. (B) Down-regulation of CD4. A flow cytometric assay was used to measure the activity of the Nef proteins for the down-regulation of CD4 from the cell surface. Cells (HEK 293) were cotransfected with plasmids expressing CD4, wild-type Nef, or the indicated Nef proteins (pCIneo-based subgenomic constructs) and GFP and then stained with an antibody to CD4 conjugated to PE and analyzed by two-color flow cytometry as described previously (7). The mean CD4 (PE) fluorescence intensity of the GFP-positive cells is graphed for two concentrations of Nef expression plasmid. Each transfection was normalized for total DNA with the empty vector (pCIneo). LL/AA encodes alanine substitutions of the leucines in the EXXXLL motif. WT, wild type.

chain, which binds to intact AP-1 and AP-2 in affinity chromatography experiments but has not been tested with AP-3 (12). The DKQTLL sequence in isolation does not interact detectably with μ subunits in yeast two-hybrid assays (39); the other leucine-based motifs have not been tested in such assays.

**Nef proteins containing either tyrosine- or leucine-based motifs are well expressed.** The mutations described above were transferred into a complete proviral plasmid clone. Cells (HEK 293) were transfected with each of the mutant proviruses and analyzed by immunoblotting for Nef expression (Fig. 1A). All of the Nef proteins were similarly expressed at steady state, allowing their biological activities to be directly compared.

**Nef proteins containing leucine motifs down-regulate CD4, whereas those containing tyrosine motifs are inactive.** The down-regulation of cell surface CD4 by Nef is a leucine-dependent and presumably AP-dependent function. To assess the ability of the various AP-binding motifs to support this function, cells (HEK 293) were cotransfected with a Nef expression plasmid, a CD4 expression plasmid, and a GFP expression plasmid and then stained with PE-conjugated antibody to CD4 and analyzed by flow cytometry. Figure 1B shows the mean PE (CD4) fluorescence of the GFP-positive cells for each transfection, with two amounts of Nef expression plasmid for each construct. Nef proteins containing leucine motifs from either tyrosinase (EERQPLL) or the TCR γ chain (DKQTLL) were fully active, the Nef protein containing the leucine motif from LIMPII (DERAPLI) was slightly impaired but active, but the Nef proteins containing the tyrosine motif from either TGN38 (SDYQRL) or LAMP 1 (AGYQTI) were devoid of activity. These results indicated that down-regulation of CD4 from the cell surface could be mediated by a variety of leucine-based sequences but not by tyrosine-based sequences. Notably, all of the Nef proteins above were equally able to down-regulate MHC class I from the cell surface, a result consistent with the dispensability of the native leucine motif for this function (data not shown) (47).

**Chimeric transmembrane proteins containing Nef as the cytoplasmic domain are down-regulated from the cell surface less efficiently if they contain a tyrosine motif.** The expression of transmembrane proteins at the cell surface correlates inversely with the activity of internalization motifs in their cytoplasmic tails. Here, chimeras consisting of the surface and transmembrane domains of the CD8 α chain fused to Nef as the cytoplasmic domain (CD8-Nef) were used to directly assess the relative activities of the Nef proteins in directing internalization. Cells (HeLa 1022) were transfected with plasmids expressing the chimeras, and the level of CD8 at the cell surface was detected at steady state by flow cytometry (Fig. 2). Wild-type CD8-Nef was expressed inefficiently at the cell surface, as were each of the mutants containing a leucine-based motif, consistent with localization to internal membranes (see Fig. 6 and 7 for confirmation of internal expression). The alanine-substitution mutant CD8-NefLL/AA (ENTSAA) was expressed approximately 10-fold more abundantly at the cell surface than the wild type (based on mean fluorescence values), reflecting loss of the internalization activity of the leucine motif (see also Fig. 6). The CD8-Nef-SDYQRL mutant was expressed approximately threefold more abundantly at the cell surface than the wild type, whereas the CD8-Nef-AGYQTI mutant was expressed approximately sevenfold more abundantly (see also Fig. 6 and 7). These data indicated that the tyrosine motifs were less active than the leucine motifs in directing the internalization of CD8-Nef, but they were not as inactive as the ENTSAA sequence. Consequently, for the Nef proteins containing the tyrosine-based motifs, there is a potential disconnect between the complete inability to down-regulate CD4 (Fig. 1) and the partial ability to mediate internalization of the CD8-Nef chimera (Fig. 2).

**Nef proteins containing leucine motifs enhance viral infectivity and growth rate, whereas those containing tyrosine motifs are virologically inactive.** The relationship between the effect of Nef on the infectivity of cell-free virions and its ability to enhance viral growth rate in cultures of human lymphocytes is complex and may involve a causal relationship with the down-regulation of CD4 during viral morphogenesis. Never-

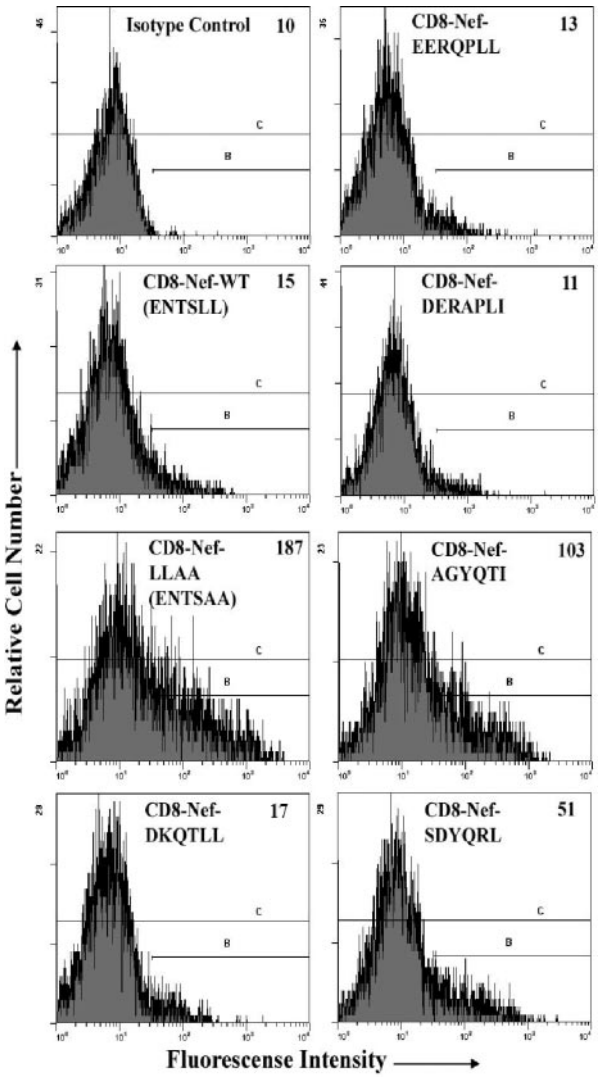


FIG. 2. Expression of CD8-Nef chimeras at the cell surface. A flow cytometric assay was used to measure the expression at the cell surface of chimeric proteins containing the surface and transmembrane domains of the CD8 α chain fused to Nef as the cytoplasmic domain. HeLa cells were transfected with plasmids expressing the various chimeras (4 μg) and then stained 24 h later with an antibody to CD8 conjugated to FITC (all panels except upper left). Cells transfected with CD8-Nef- LL/AA were used for the isotype control (upper left panel). Gate B shows the CD8-positive cells. The number in the upper right of each panel is the mean fluorescence intensity of all cells (gate C).

theless, CD4-negative cells still reveal the effect of Nef on infectivity when used as virus producers (36). To examine the activities of the AP-binding motifs in the CD4-independent effect, *nef* mutants were produced by transfection of CD4-negative HEK 293 cells, and the virions were used to infect HeLa-CD4 cells that contain a long terminal repeat-driven β-galactosidase indicator. Infectivity was determined by counting the infectious centers (Fig. 3A). Nef-negative and ENT SAA (NefLL/AA) mutant viruses were less infectious than the wild type, in agreement with previously reported data (7). Notably, in these experiments with CD4-negative producer cells, the infectivity defect of the NefLL/AA mutant was not as great as the *nef*-defective virus. Although their infectivity was not fully restored to that of the wild type, mutants encoding the substituted leucine motifs were more infectious than the *nef*-negative or NefLL/AA mutants. In contrast, the mutant encoding the SDYQRL sequence was as impaired as NefLL/AA, and the mutant encoding the AGYQTI sequence was almost as impaired as NefLL/AA. Together, these data indicated that the CD4-independent effect of Nef on viral infectivity could be mediated by a variety of leucine-based sequences, whereas the tyrosine-based sequences had little or no activity.

These virologic data were extended to include the role of CD4 during virion production and the use of primary human lymphocytes as host cells. Viruses were produced from cocultures of CD4-positive CEM T cells together with HEK 293 cells that had been transfected transiently with the proviral constructs. This coculture method allowed the production of high-titer viral stocks from CD4-positive T cells with a short (48 h) interval of virion production. The growth rates of these viruses were measured by using cultures of PBMCs that were stimulated with phytohemagglutinin and interleukin-2 before infection (Fig. 3B). The mutants encoding leucine motifs grew at a rate identical to that of the wild type, whereas the mutants encoding tyrosine motifs grew at reduced rates identical to that of LL/AA mutant virus. These data confirmed that a variety of leucine-based AP-binding motifs are virologically active when inserted within the Nef C-terminal loop, whereas tyrosine-based motifs are virologically inactive.

**Nef proteins containing either tyrosine- or leucine-based motifs interact with the μ subunits of AP complexes.** The interactions of the Nef proteins with the μ subunits of AP-1 (μ1), AP-2 (μ2), and AP-3 (μ3) were analyzed by using a quantitative yeast two-hybrid assay (Fig. 4). Each Nef protein was expressed as a fusion with the DNA binding domain of the Gal4 transcriptional activator, and each of the μ subunits was expressed as a fusion with the activation domain of Gal4. In yeast cells cotransformed with plasmids expressing the Nef and μ fusion proteins, interaction causes transcriptional activation of the *HIS3* reporter gene. The rate of yeast growth in media lacking histidine reflects the avidity of the interaction between Nef and the μ subunits. As previously reported, wild-type Nef interacted most avidly with μ1, next most avidly with μ3, and very weakly with μ2 (8).

Compared to the wild-type, Nef proteins containing tyrosine-based sequences showed altered relative avidities for μ1, μ2, and μ3 (Fig. 4). The Nef protein containing the sequence from TGN38, SDYQRL, interacted equally with each of the μ subunits, in agreement with data in which the isolated motif was tested with these μ chains in yeast two-hybrid assays (11, 38, 39). The Nef protein containing the tyrosine motif from the LAMP I, AGYQTI, interacted best with μ3, again in agreement with two-hybrid data regarding the μ-binding preferences of the isolated motif (51). Together, these data indicated that the tyrosine-based sequences were active within the context of the C-terminal loop of Nef because they modulated μ binding in a predictable manner. Strikingly, the inability of the tyrosine-based sequences to support Nef function (Fig. 1 and 3), despite their ability to bind the μ subunits (Fig. 4), indicates that μ binding is a poor functional correlate and is likely insufficient to confer Nef activity.

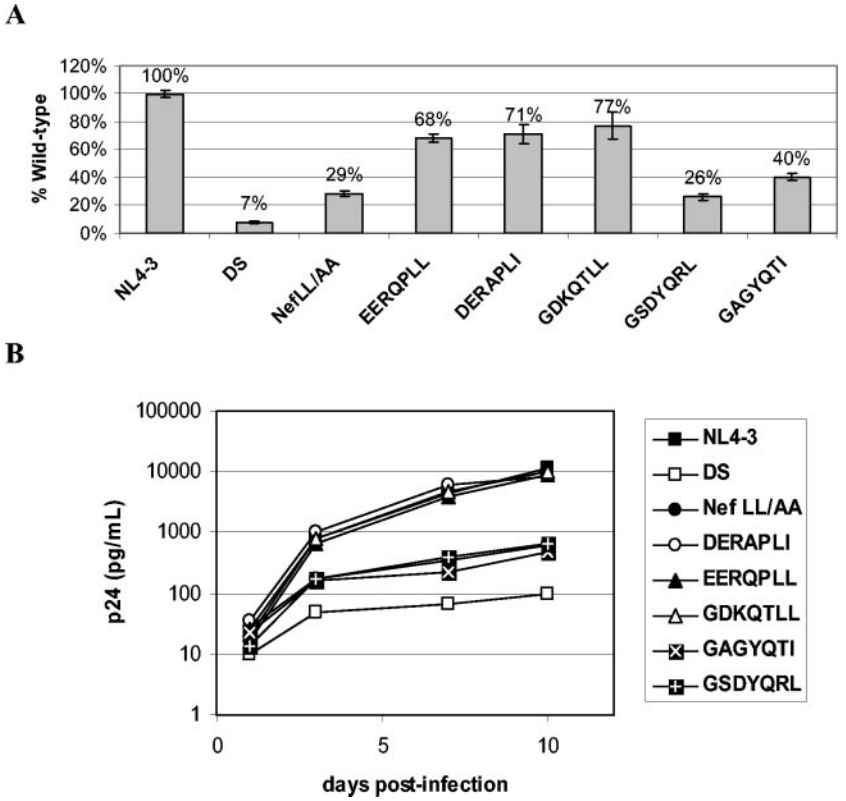


FIG. 3. Virologic analysis of *nef* mutants. (A) Viral infectivity of *nef* mutants in an infectious center assay. Cell-free virions were produced by transient transfection of CD4-negative HEK 293 cells and then used to infect CD4-positive HeLa cells as described in Materials and Methods. Infectious centers were counted, the infectivity of each viral stock was expressed as the ratio of infectious centers to picograms of p24 (capsid) antigen, and the data were expressed as percentages of infectivity (that of wild-type virus was normalized to 100%). NL4-3 is wild-type virus; DS is *nef*-negative virus (containing two N-terminal premature stop codons) (6). The AP-binding sequence in the Nef protein of each virus is indicated; NefLL/AA is the alanine substitution mutant of the LL sequence in the native Nef motif. Data are the averages of the results of duplicate experiments; error bars represent standard deviations. (B) Viral growth rate of *nef* mutants in cultures of PBMCs. Viruses were produced from cocultures of transfected 293 cells and CD4-positive CEM T cells. PBMCs were activated with phytohemagglutinin and interleukin-2 and then infected as described in Materials and Methods. The concentration of p24 (capsid) antigen in the cultures is shown over time.

In contrast to the tyrosine-based sequences, the various leucine-based sequences each supported a binding hierarchy that was similar to that of wild-type Nef: $\mu1 > \mu3 > \mu2$. The binding of Nef-DERAPLI to $\mu1$ and $\mu3$ but not to $\mu2$ matched the AP-binding properties of the isolated motif (15, 22). However, the EERQPLL sequence supported binding to both $\mu1$ and $\mu3$ in the context of Nef, whereas only binding to intact AP-3 has been reported for the isolated motif (23). No data on the binding of the TCR $\gamma$ chain to AP3 are available to corroborate the ability of the DKQTLL sequence to support binding to $\mu3$ in the context of Nef. However, the weak binding of Nef-DKQTLL to $\mu2$ was in conflict with the reported binding of the DKQTLL sequence to intact AP-2 (12). In general, the substitution of heterologous leucine-based sequences did not alter dramatically the $\mu$ binding hierarchy of Nef, suggesting that this subunit may not be the target of these sequences.

**Leucine-based but not tyrosine-based motifs support formation of a ternary complex involving Nef and either the $\sigma1$ plus $\gamma$ subunits of AP-1 or the $\sigma3$ plus $\delta$ subunits of AP-3.** The data above suggested that the binding of Nef to the $\mu$ subunits, as modulated by tyrosine-based motifs, was insufficient for biologic function. To determine whether this lack of function was associated with inefficient interaction with the entire complex, binding to intact AP complexes from cytoplasmic lysates was tested with a GST pull-down assay. The Nef protein containing the SDYQRL sequence was studied because it bound well to $\mu1$ in the yeast two-hybrid assay yet was devoid of biologic activity. As a GST fusion protein, Nef-SDYQRL bound inefficiently to intact AP-1 from cytoplasmic lysates (Fig. 5A). The extent of binding to AP-1 by Nef-SDYQRL was above the background binding for GST alone or GST-Nef containing alanine substitutions of the leucines in the native motif, but it was less than 20% that of wild-type Nef. These data confirmed that the binding to $\mu1$ as provided by a tyrosine-based motif is insufficient for the efficient interaction of Nef with intact AP-1.

The data above suggested that leucine motifs support an especially avid binding to AP complexes when within Nef that is neither provided by tyrosine motifs nor is likely due solely to an interaction with the $\mu$ subunits. Consequently, potential interactions with the other subunits of the complex were evaluated. The $\beta$ subunit has been proposed as a binding target for leucine-based motifs based on chemical cross-linking studies (46), and a leucine-dependent binding between Nef and the 171 to 518 fragment of $\beta1$ was described by the two-hybrid assay (16). However, we did not observe a consistent interaction between Nef and $\beta1$ (data not shown). Similarly, we did

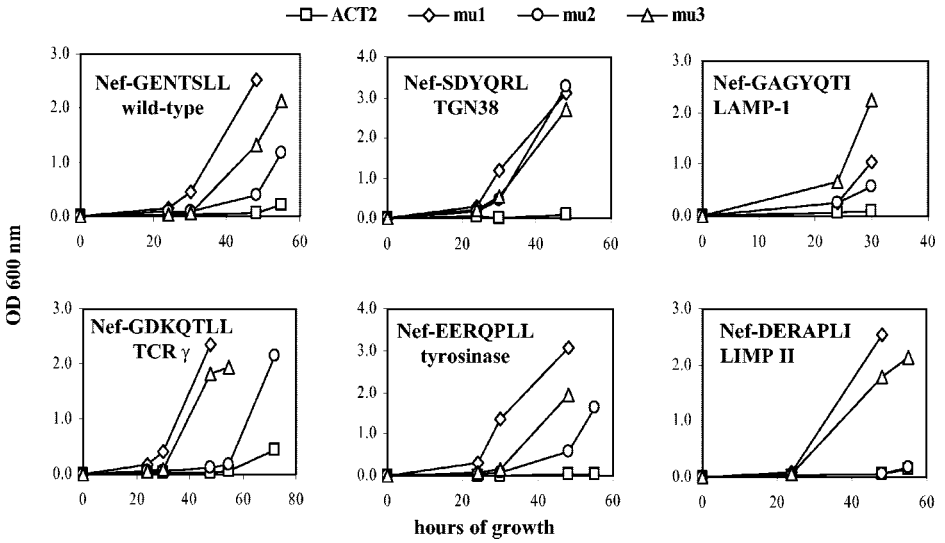


FIG. 4. Interactions between Nef proteins and μ subunits of AP complexes assessed by quantitative yeast two-hybrid assays. Wild-type Nef or mutated Nef proteins containing the indicated sequences were fused to the DNA-binding domain of the Gal4 transcriptional activator encoded by the plasmid pGBKT7. These plasmids were used to cotransform yeast along with pACT2-based plasmids expressing each of the μ subunits fused to the activation domain of Gal4. Colonies of cotransformed yeast were pooled as described in Materials and Methods and then used to inoculate liquid cultures in media lacking histidine. The growth rates of the yeast cells were measured as the optical density (OD) at 600 nm over time and reflect interaction of the two hybrids, transcription of the *HIS3* gene, and histidine biosynthesis. ACT2 is the parental plasmid encoding only the activation domain of Gal4 and provides the background for each Nef fusion protein. The sequences of the AP-binding sequences within the C-terminal loop of Nef and their proteins of origin are indicated in the upper left of each panel. Datum points are the averages of results from duplicate experiments. The results are representative of two independent yeast transformations.

not detect an interaction between wild-type Nef and γ adaptin, the large, specific subunit of AP1 (data not shown). Together, these data suggested that HIV-1 Nef interacts either with multiple subunits of the AP complex simultaneously or with a single subunit whose correct conformation is dependent on interactions within the intact complex.

In support of this hypothesis, recent yeast three-hybrid data have revealed a leucine-dependent ternary interaction between Nef and AP hemicomplexes containing two of the four subunits: the σ1 plus γ subunit of AP-1 and the σ3 plus δ subunit of AP-3 (25). In this assay, one yeast vector expressed a Nef-Gal4 DNA-binding domain fusion along with a σ subunit as the bridge protein, while the other vector expressed the large, specific subunit of the complex (γ or δ) as a Gal4 activation domain fusion. As in the two-hybrid assay, yeast cells cotransformed with the two vectors grow on media lacking histidine only if a protein-protein interaction reconstitutes Gal4-mediated transcription of the *HIS*3 gene. As previously shown (25), wild-type Nef interacted in a leucine-dependent manner with the σ1 plus γ subunits of AP-1 and the σ3 plus δ subunits of AP-3 (Fig. 5B). No interaction was detected between Nef and the μ1 plus β1 subunits of AP1 (data not shown). The Nef proteins that contain tyrosine-based sequences bound neither the σ1-γ hemicomplex of AP-1 nor the σ3-δ hemicomplex of AP-3 (Fig. 5B), supporting the leucine specificity of this interaction. However, only two of the three heterologous leucine-based motifs supported the interaction between Nef and the σ1-γ and/or σ3-δ hemicomplexes: Nef-EERQPLL interacted with both hemicomplexes, Nef-DERAPLI interacted only with the σ1-γ hemicomplex, and Nef-DKQTLL interacted with neither hemicomplex (Fig. 5B).

These data established the binding of Nef to the σ1-γ or σ3-δ hemicomplex as a better correlate of function than binding to the μ subunits, but they left unexplained the function of the Nef-DKQTLL protein (Fig. 1B and 3). Consequently, we investigated whether interactions with other components of the endosomal sorting machinery may correlate more precisely with function. The β subunit of the COPI vesicle coat and the catalytic subunit (H) of a human vacuolar ATPase homologue have each been reported to bind to HIV-1 Nef (2, 31); these interactions reportedly require distinct acidic residues that flank the leucine-based sorting motif within the C-terminal loop (31, 43). Each of the Nef proteins herein interacted similarly with either β-COP or the vATPase H subunit in yeast two-hybrid assays, regardless of whether they contained tyrosine-based or leucine-based motifs (data not shown). We also considered that Nef may bind a member of the GGA family of coat proteins; these are monomeric adaptors that bind acidic cluster leucine motifs such as that found in the cytoplasmic domain of the mannose-6 phosphate receptor (45). Neither GGA-1, -2, nor -3 bound detectably to wild-type Nef in the yeast two-hybrid assay, whereas each GGA protein bound the cytoplasmic tail of the mannose-6 phosphate receptor (data not shown). Together, these data suggested that the GGA proteins, β-COP, and the vATPase H subunit are not relevant partners of the leucine-based motif in Nef.

**CD8-Nef chimeras containing leucine-based motifs stabilize AP-1 and AP-3 on juxtanuclear membranes, but those containing tyrosine-based motifs do not.** The data of Fig. 1, 3, and 5A indicate that the function of Nef specifically requires leucine-mediated binding to AP complexes. We recently described a leucine-dependent ability of HIV-1 Nef to stabilize the association of AP-1 and AP-3 on juxtanuclear membranes by an ARF1-independent mechanism that is insensitive to

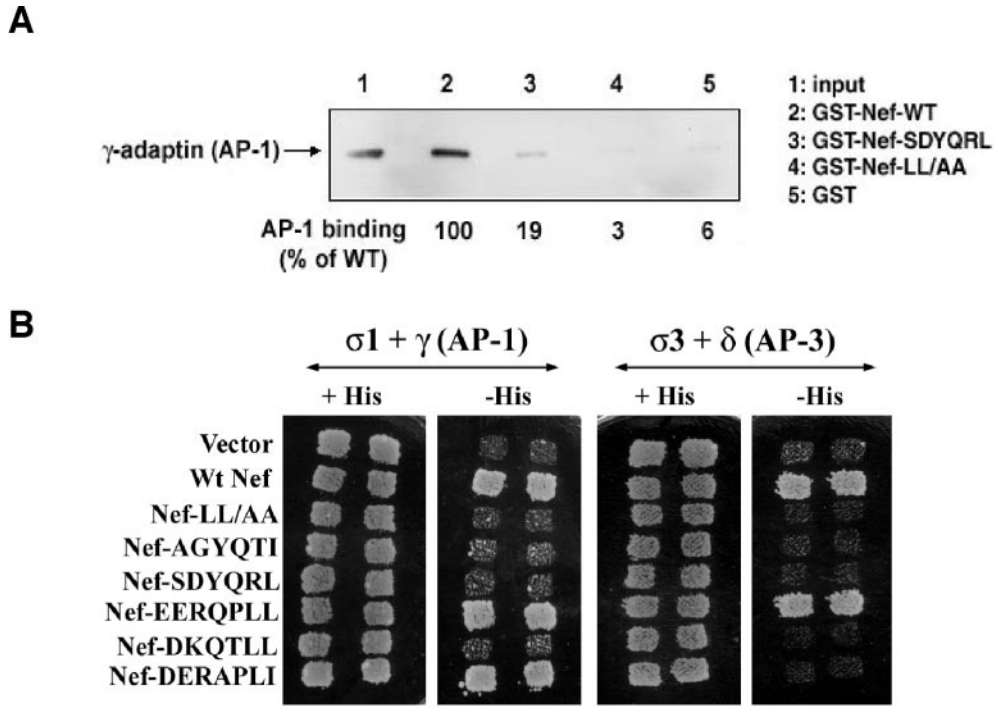


FIG. 5. Leucine-dependent interactions of Nef with intact AP complexes and with hemicomplexes containing the small subunit plus the large, specific subunit. (A) Interaction with intact complexes. Equal amounts of purified GST-Nef, GST-Nef-SDYQRL, GST-Nef-LL/AA, and GST were immobilized on glutathione-agarose beads and then incubated with cytoplasmic lysates from HeLa cells. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and AP-1 was detected by Western blotting with an antibody to γ-adaptin. Bands were quantified by using NIH Image software; the numerical data are expressed as percentages of binding relative to wild-type (WT) Nef. Input is an unfractionated HeLa cell lysate used as a control for the detection of γ-adaptin; the amount loaded is much less than the amount of lysate used in the incubations with the GST-agarose beads. (B) Interaction with AP hemicomplexes by yeast three-hybrid assay. Wild-type Nef or mutated Nef proteins were fused to the DNA-binding domain of the Gal4 transcriptional activator encoded by the plasmid pBridge, which also encoded either the σ1 or σ3 subunit. These plasmids were used to cotransform yeast along with pGADT7-based plasmids expressing either the γ or δ subunits fused to the activation domain of Gal4. Colonies of cotransformed yeast were patched and replica plated onto media containing or lacking histidine, as described in Materials and Methods and then incubated for 24 to 48 h at 30°C. As in the two-hybrid assays, growth on media lacking histidine indicates a protein-protein interaction. Vector is the pBridge plasmid encoding neither Nef nor a σ subunit and provides the background for the γ-Gal4 and δ-Gal4 activation domain fusion proteins. The results are representative of two independent yeast transformations.

brefeldin A (BFA) (24). Treatment of cells with BFA prevents the attachment of AP-1 and AP-3 to membranes by inhibiting guanine nucleotide exchange factors for the GTP-binding protein ARF-1 (13). During a short incubation of living cells with BFA (2 to 15 min), the complexes cycle off membranes and become diffusely cytosolic. Nef has the unusual ability to induce the persistent association of AP-1 and AP-3 with juxtanuclear membranes despite this BFA-induced block to attachment. To determine whether this Nef activity is leucine specific and correlates with function, we analyzed the distribution of AP-1 and AP-3 in BFA-treated cells that expressed CD8-Nef chimeras in which Nef constitutes the cytoplasmic domain (Fig. 6 and 7). AP-1 was stabilized on juxtanuclear membranes by every Nef protein that contained a leucine-based motif (Fig. 6). In contrast, neither of the Nef proteins that contained a tyrosine-based motif stabilized the association of AP-1 with juxtanuclear membranes, despite the presence of the chimeras in this region (Fig. 6). Similarly, Nef proteins containing a leucine motif stabilized the membrane association of the AP-3 complex, whereas those containing a tyrosine motif did not (Fig. 7). The EERQPLL sequence from tyrosinase and the DERAPLI sequence from LIMP II were particularly robust, consistent with their preferential binding to AP-3 in vitro (22). These data indicated that the membrane stabilization of AP-1 and AP-3 by Nef specifically requires leucine-based motifs; the data also indicated that these in vivo interactions correlate with Nef function.

## DISCUSSION

We have replaced the AP complex-binding motif in HIV-1 Nef with distinct motifs from cellular proteins and correlated protein interactions with biologic function. The C-terminal loop of Nef provided a structurally favorable context for the presentation of a variety of AP-binding motifs; the insertion of tyrosine-based motifs into this location altered the μ-binding specificities of the complete Nef protein to match those reported for the motifs in isolation (11, 38, 39). In contrast, leucine-based motifs did not alter significantly the hierarchy of interactions between Nef and the μ subunits, suggesting that the μ subunit is not their binding partner. Strikingly, every Nef protein that contained a leucine-based motif down-regulated CD4 and enhanced viral infectivity and replication, whereas those that contained a tyrosine-based motif were inactive.

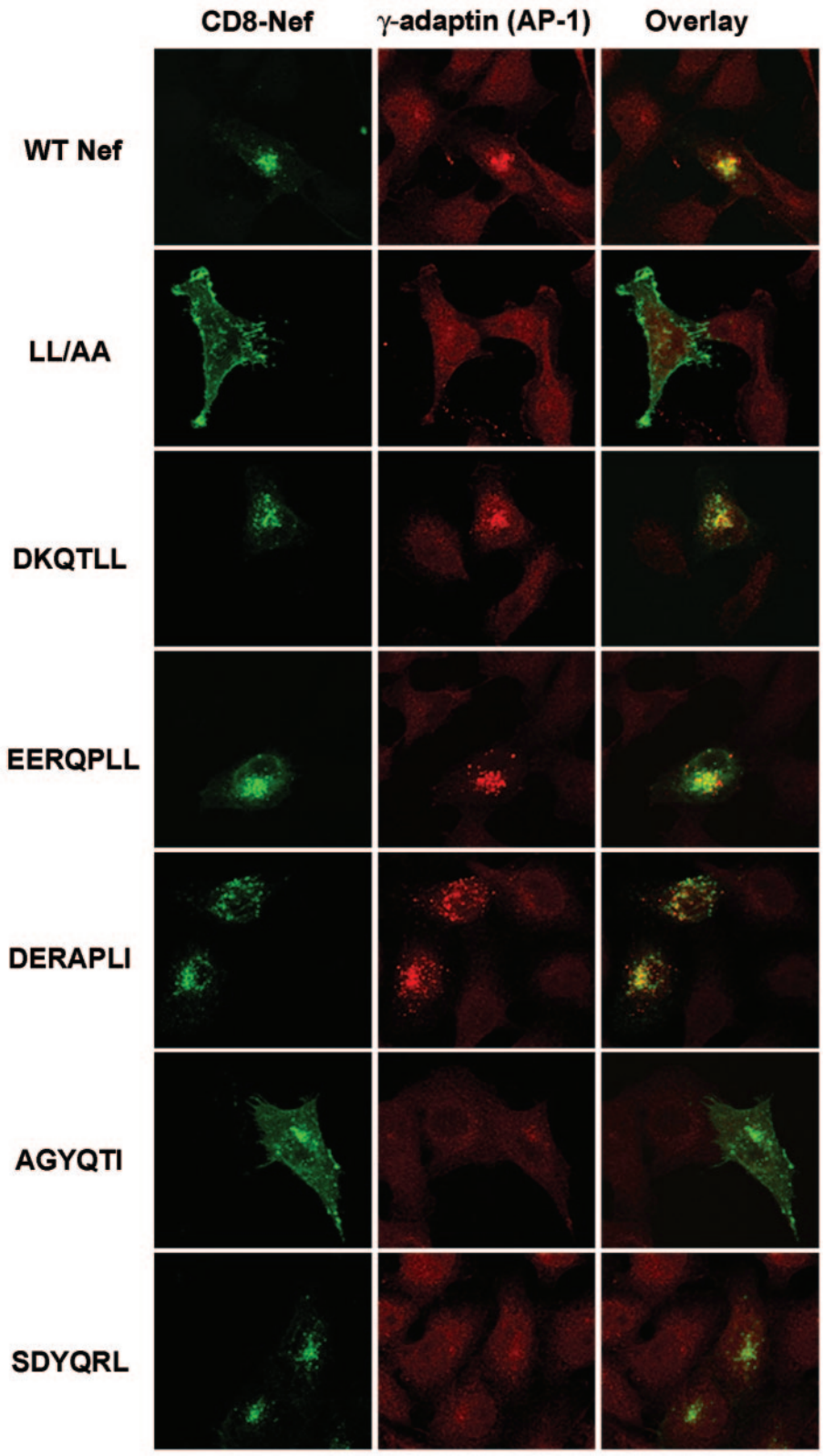


FIG. 6. Stabilization of AP-1 by Nef proteins containing leucine- but not tyrosine-based motifs. Chimeric proteins containing the lumenal and transmembrane domains of the CD8 α chain and either wild-type Nef or the mutated Nef proteins as the cytoplasmic domain were expressed by transient transfection in HeLa cells. To identify AP complexes that were specifically stabilized by Nef, the cells were treated with 10 μg of BFA/ml for 15 min before fixation, staining, and analysis by confocal immunofluorescence microscopy. The distribution of the chimeras within the cells was analyzed in relation to AP-1. Green, CD8; red, the γ subunit of AP-1; WT, wild type; LL/AA, the alanine substitution mutant of the leucines in the native Nef motif. The remaining mutants are identified by their sequences.

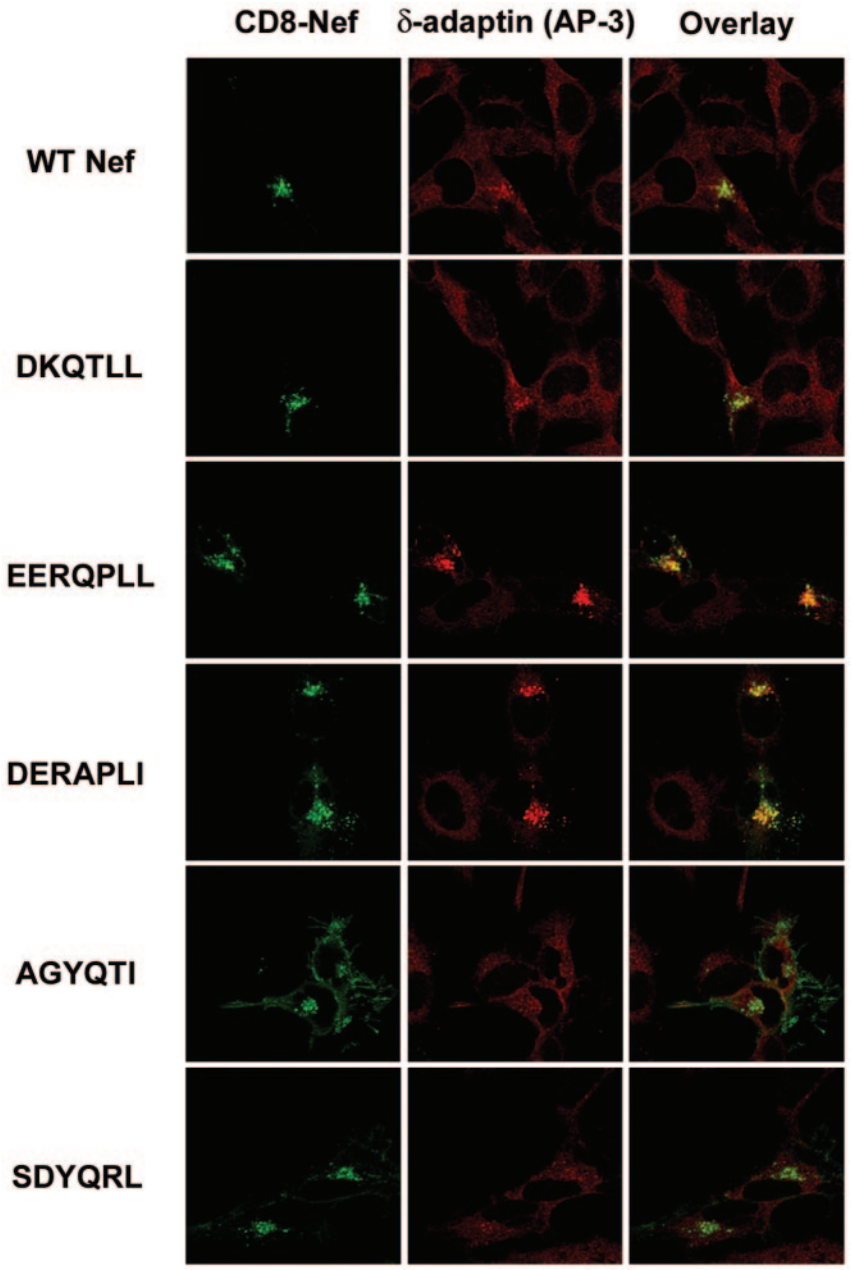


FIG. 7. Stabilization of AP-3 by Nef proteins containing leucine- but not tyrosine-based motifs. CD8-Nef chimeras were expressed by transient transfection in HeLa cells, and their distribution was analyzed in relation to AP-3 after treatment with BFA as described in the legend to Fig. 6. Green, CD8; red, the δ subunit of AP-3; WT, wild type.

These data indicated that the Nef-μ interaction is insufficient for function. The interaction of Nef with hemicomplexes of AP-1 (σ1 plus γ) and/or AP-3 (σ3 plus δ) correlated more closely with function, but it was not a fully sensitive predictor of Nef activity. Every heterologous leucine-based motif but neither of the tyrosine-based motifs supported the BFA-resistant association of AP-1 and AP-3 with juxtanuclear membranes, indicating that the recruitment and stabilization of AP-1 and AP-3 on membranes in vivo correlated closely with Nef function.

Are hemicomplexes containing the small plus large, specific subunits of AP complexes unequivocally the targets of leucine motifs? This interaction was both leucine dependent and leucine specific. However, it was an imperfect correlate of function within the context of Nef; the Nef-DKQTLL mutant interacted with neither the AP-1 nor AP-3 hemicomplex, yet its functional phenotype was nearly wild type. Conceivably, Nef-DKQTLL may interact with another AP hemicomplex, for example the σ2 plus α subunits of AP-2. Alternatively, the sensitivity of the three-hybrid assay may be insufficient to detect every functional interaction. Correlation of the hemicomplex interactions with the stabilization of AP complexes in vivo

provides evidence of such a sensitivity problem; for example, Nef-DKQTLL did not bind to the AP-1 or AP-3 hemicomplex, but it stabilized AP-1 and AP-3 on membranes in vivo. Similarly, Nef-DERAPLI did not bind the AP-3 hemicomplex, but it stabilized AP-3 on membranes in vivo. Despite these caveats, the data herein support the contention that the hemicomplex interaction is leucine dependent and not supported by tyrosine-based motifs (25). Consequently, these data support a model in which tyrosine- and leucine-based motifs bind by distinct mechanisms to AP complexes (34).

In the context of Nef, the AP binding provided by the interaction of tyrosine-based motifs with μ subunits seems inefficient when compared to the AP binding provided by leucine-based motifs. Nevertheless, the binding between native Nef and the μ subunits may be functionally relevant. We reported previously that the interaction between Nef and μ1 depends on sequences in the structured regions of Nef that flank the solvent-exposed, C-terminal loop, which contains the leucine motif (14). These flanking regions appear to play a significant role in the trafficking and function of Nef because they are required for the efficient internalization of transmembrane chimeras and for the down-regulation of CD4. They are also required for the stabilization of AP-1 on membranes (data not shown). In addition, we have observed that wild-type Nef fails to bind μ1 mutants encoding alanine substitutions of either residues D174 or V392 and L395 (data not shown); these residues are analogous to those that define the binding pocket for tyrosine-based motifs in μ2 (40). This indicates that part of Nef, presumably the residues flanking the loop, interacts with the μ subunits as if it contained a tyrosine-based motif.

Together, these observations support a model in which Nef binds the AP complexes via two interactions: one between sequences that flank the loop and the μ subunit and the other between the leucine motif and a second site on the complex. This second, leucine-dependent site is likely formed by the small subunit plus the large, specific subunit (25; data herein). In this model, substitution of a tyrosine-based motif for the leucine-based motif in Nef replaces the leucine-hemicomplex interaction with an interaction that the protein essentially already has. The uncompensated loss of the leucine-based interaction reduces dramatically the affinity of Nef for the intact complex, with consequent loss of biologic function.

What are the roles of specific AP complexes in Nef function? The AP-2 complex is widely assumed to mediate Nef-induced endocytosis of CD4 (42). However, in contrast to AP-1 (and AP-3), the interaction of HIV-1 Nef with intact AP-2 in vitro is barely detectable, and microscopic overlap of Nef with AP-2 is not apparent with the fully functional allele used here (4, 8). The data herein indicate that the interaction of Nef with the μ subunit of AP-2 is insufficient for function. However, Nef-DERAPLI is slightly impaired in its ability to down-regulate CD4, and it is the only mutant that has virtually no detectable binding to μ2 in the two-hybrid assays. Consequently, it is possible that binding to μ2 (and AP-2), while insufficient, may still contribute to Nef function. Furthermore, leucine-mediated binding to the AP-2 hemicomplex cannot be excluded; we are presently unable to confirm the lack of such binding due to apparent toxicity of the AP-2-related constructs in the three-hybrid assay. Although uncertainty persists regarding the role of AP-2, the data herein support the hypothesis that a primary action of HIV-1 Nef is postendocytic. Postendocytic targeting of CD4 from early endosomes to late endosomes and lysosomes is consistent with the use of AP-1 and AP-3 as Nef cofactors. Indeed, two of the leucine motifs that supported Nef function, DERAPLI and EERQPLL, interact in vitro with intact AP-3 and/or AP-1 but not with AP-2 (22). These data suggest that within the family of AP complexes, AP-1 and/or AP-3 may be sufficient cofactors for the Nef-mediated down-regulation of CD4.

Strikingly, the data herein provide a close correlation between the functions of Nef and its ability to stabilize the AP-1 and AP-3 complexes on endosomal membranes. This ARF1-independent stabilization correlates with the direct binding of Nef to intact complexes in vitro (24). The data herein show that the Nef-mediated stabilization strictly requires a leucine motif; it is both leucine dependent and leucine specific. This membrane stabilization is likely a consequence of an unusually avid binding to Nef, requiring both the leucine-based hemicomplex interaction and the interaction with the μ subunit as described in the model above.

How does the AP stabilization phenomenon relate to Nef function? The persistent attachment of AP complexes to membranes seems likely to cause a general distortion of the endosomal system, which may account for the influence of Nef on several cellular proteins (24). Mechanistically, membrane stabilization of the complexes may also cause proteins to become recruited into coated vesicles despite a relatively low affinity for AP complexes. In this model, Nef would facilitate the interaction of target proteins with AP complexes by stabilizing the formation of the vesicle coat, rather than serving as a simple connector between the target protein and the complex. Support for this model derives from the data of Fig. 1 and 2 and is exemplified by the Nef-SDYQRL mutant. This mutant has significant internalization activity when fused to CD8, but it is devoid of activity in down-regulating CD4. In the connector model, this phenotype indicates a defect in binding to CD4 (33). However, in the case of Nef-SDYQRL (and Nef-AGYQTI) this seems unlikely, because the putative CD4-binding site on HIV-1 Nef is formed by residues in the N-terminal half of the protein and is completely distinct from the site of these mutations, the C-terminal loop (19). Conceivably, these mutants could fail to bind CD4 due to a misfolding artifact. Alternatively, their phenotypes may confirm a distinction suggested by the two-hybrid, GST pull-down, and in vivo recruitment data: Nef function seems to require not merely the binding to AP complexes but also the ability to stabilize the complexes on membranes. Indeed, the Nef-SDYQRL and Nef-AGYQTI mutants, though apparently able to interact with AP complexes sufficiently well to be internalized, fail to stabilize the complexes on membranes and are unable to down-regulate CD4. Consequently, these data support a scenario in which the critical Nef activity is the stabilization of coat formation and, presumably, consequent facilitation of the inclusion of target proteins such as CD4 into endosomal vesicles.

In summary, the activity of HIV-1 Nef requires a specific mode of interaction with AP complexes provided only by leucine motifs. This mode of interaction likely involves a ternary complex between Nef and the small plus large, specific AP subunits. These observations, together with previous data on the molecular basis of the interaction of Nef with μl (14), lead

to a model in which Nef binds AP complexes via two distinct interactions: one between sequences that flank the C-terminal loop and the μ-subunits and the other between the leucine-based motif within the loop and the small plus large, specific subunits. We propose that these two interactions cooperate to yield the high-affinity binding required for the recruitment and stabilization of AP complexes on membranes. We further propose that stabilization of the vesicle coat is central to the mechanism by which Nef disregulates the endosomal trafficking of CD4 and other transmembrane proteins.

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