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The Human Thioesterase II Protein Binds to a Site on HIV-1 Nef Critical for CD4 Down-regulation*

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A HIV-1 Nef affinity column was used to purify a 35-kDa Nef-interacting protein from T-cell lysates. The 35-kDa protein was identified by peptide microsequence analysis as the human thioesterase II (hTE) enzyme, an enzyme previously identified in a yeast two-hybrid screen as a potential Nef-interacting protein. Immunofluorescence studies showed that hTE localizes to peroxisomes and that coexpression of Nef and hTE leads to relocalization of Nef to peroxisomes. Interaction of Nef and hTE was abolished by point mutations in Nef at residues Asp^{108}, Leu^{112}, Phe^{121}, Pro^{122}, and Asp^{123}. All of these mutations also abrogated the ability of Nef to down-regulate CD4 from the surface of HIV-infected cells. Based on the x-ray and NMR structures of Nef, these residues define a surface on Nef critical for CD4 down-regulation. A subset of these mutations also affected the ability of Nef to down-regulate major histocompatibility complex class I. These results, taken together with previous studies, identify a region on Nef critical for most of its known functions. However, not all Nef alleles bind to hTE with high affinity, so the role of hTE during HIV infection remains uncertain.

The *nef* gene of the human immunodeficiency virus (HIV)[1] encodes a 27-kDa myristoylated cytosolic protein that associates with the plasma membrane and other intracellular vesicle surfaces (1–3). Although the precise functions of Nef remain controversial, in SIV-infected adult macaques Nef expression is critical for the maintenance of a high viral load and progression to AIDS (4). Moreover, in humans, several long term survivors of HIV infection carry HIV with deletions in *nef* or with a high frequency of defective *nef* alleles (5–7).

How Nef achieves these *in vivo* effects is not yet clear. However, in tissue culture a number of effects of Nef have been documented. First, it enhances viral infectivity and replication in primary cells (8, 9). Second, it alters the state of T-cell activation and macrophage signal transduction pathways (10–13). Third, it reduces the cell surface expression of CD4, one of the cellular receptors for HIV (1–3, 14). The internalization and degradation of cell surface CD4 by Nef increases the infectivity of the released virion particles because CD4 interferes with incorporation of the HIV envelope protein into the virus particle (15, 16). Finally, Nef also down-regulates cell surface expression of MHC class I. This effect may be important for HIV immune evasion (17–20). Because Nef has no known catalytic activity, the above activities are probably mediated through interaction of Nef with host cell proteins, and several cellular proteins have been suggested to interact with Nef. For example, Nef contains a consensus SH3 domain binding sequence (P*XX*P) that mediates Nef association with members of the Src tyrosine kinase family (21–24). Nef also associates with components of the endocytic machinery, β-cop, and the clathrin adaptor complex, and these interactions are important for linking CD4, through Nef, to endocytic pathways (19, 25–29). Nef has also been reported to interact with a member of the p21-activated kinase family (30, 31) and a vacuolar ATPase (32).

Recently, another Nef-interacting protein, human thioesterase (hTE), was cloned from a Jurkat T-cell cDNA library in a yeast two-hybrid screen (33, 34). hTE belongs to a novel class of thioesterases and exhibits 42% amino acid identity with an *Escherichia coli* thioesterase II (35). The precise biological function of either hTE or its *E. coli* homolog is not yet clear. The best characterized thioesterases are involved in lipid metabolism; yet overexpression or deletion of the bacterial TEII enzyme in *E. coli* had no detectable effect on fatty acids levels (35).

To investigate the role of Nef in modulating cell-signaling pathways, we used a Nef affinity column to purify Nef-interacting proteins from T-cell lysates. We found that hTE was the only protein in a T-cell lysate that associated with Nef with high enough affinity to be identified by peptide microsequence analysis in this screen. We then identified five point mutations in Nef that abolish binding to hTE. All of these mutations abrogated the ability of Nef to down-regulate CD4 from the surface of infected cells. Based on the x-ray and NMR structures of Nef, these mutations defined a surface on Nef critical for CD4 down-regulation. We found that part but not all of this region on Nef is also critical for Nef-induced MHC class I down-regulation. The mutagenesis analysis and *in vivo* immunofluorescence colocalization studies with Nef and hTE suggest that hTE plays a role during HIV infection. However, we found that not all Nef alleles bind to hTE with high affinity (*e.g.* SF2

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[1] The abbreviations used are: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; GFP, green fluorescence protein; GST, glutathione *S*-transferase; PLAP, placental alkaline phosphatase; hTE, human thioesterase II; MHC, major histocompatibility complex; Flu, influenza hemagglutinin A epitope; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

Nef and SIV Mac239 Nef). Therefore, it is uncertain whether hTE binding plays a critical role in Nef function.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The pGEX2T vector construct (Amersham Pharmacia Biotech) was used to express glutathione *S*-transferase fusion proteins of various Nef mutants. hTE cDNA containing a hexahistidine tag followed by a thrombin cleavage site was cloned into the expression vector, pRSET (Invitrogen) for expression in bacteria. Genes encoding for CD4, various Nef alleles, green fluorescence protein (GFP) fusion proteins, and N-terminally Flu-tagged hTE were cloned into the pBABE retroviral expression vector (36) for expression in mammalian cells. Nef-GFP fusion constructs contained Nef at the 5′ end, a 12-base pair linker (GGC GGC CGC AGC) and enhanced humanized GFP (CLONTECH) at the 3′ end and are similar to a previously described construct used in Nef localization studies (28).

Random Mutagenesis of Nef at Residues Leu^{76}, Pro^{78}, Asp^{108}, Leu^{112}, Tyr^{115}, Phe^{121}, Pro^{122}, and Asp^{123}—For each residue mutated we synthesized a pair of overlapping primers where the codon to be mutated was synthesized using a mixture of all four nucleotide precursors. For example, the coding strand primer for mutating NL4–3 Nef at Asp^{108} contained the sequence CAC TCC CAA AGA AGA CAA NNC ATC CTT GAT CTG TGG ATC (where that N indicates all four nucleotide bases were used). A separate polymerase chain reaction and ligation reaction was then done for each of the 8 residues to create a library of mutants at that residue. For each residue, 10 bacterial colonies were chosen at random and tested for association with hTE in the GST pull-down assay.

Purification of Recombinant GST Fusion and Nef Proteins—Bacteria containing the hTE/pRSET plasmid were induced for hTE expression by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2–6 h. Cells were harvested, lysed by sonication, and centrifuged. The supernatant was passed over a nickel-nitrilotriacetic acid column and washed with a buffer containing 90 mM imidazole followed by elution of hTE with a buffer containing 200 mM imidazole.

GST fusion proteins from bacteria were purified using glutathione-agarose beads (Molecular Probes) (24). Nef protein alone (in the absence of GST) was obtained by cleaving the thrombin cleavage site in the GST-Nef fusion protein, while the fusion protein was immobilized to the agarose beads. The eluted Nef protein was further purified by fast protein liquid chromatography over a Mono Q column (Amersham Pharmacia Biotech) followed by dialysis. This purified Nef protein was used to make a Nef affinity column using Affi-Gel beads (Bio-Rad) and for other experiments (*e.g.* circular dichroism) described in the text.

Purification of Nef-interacting Proteins from T-cell Lysates—Fresh calf thymii were washed in ice-cold PBS, cut into small pieces, and 2 volumes (weight to volume) of ice-cold buffer A (10% glycerol, 25 mM Hepes, pH 7.5, 140 mM KCl, 1.3 mM EDTA, 1.0 mM $MgCl_2$, 3.0 mM dithiothreitol) plus 0.01% aprotinin, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM KF, 0.25 mM orthovanadate, and 1 μM leupeptin were added. This mixture was pureed in a blender and sieved through a metal basket. Triton X-100 was added to 0.25%, and the mixture was stirred for 20′ at 4 °C and centrifuged 15′ at 1,500 × *g*, and the supernatant was passed through cheese cloth, recentrifuged at 20,000 × *g* for 45′, and then recentrifuged again at 80,000 × *g*. The supernatant was adjusted to 0.02% azide, held at 4 °C overnight, and respun at 80,000 × *g*. The supernatant was then precleared by passing over an Affi-Gel bead column. Affi-Gel beads containing cross-linked Nef were then added (final concentration of Nef varied between 0.1 and 1.0 μM), and the mixture was rotated overnight at 4 °C. The beads were then transferred to a column, and washes were done with Buffer A plus increasing concentrations of NaCl (up to 1.0 M). Nef-associated proteins were eluted in Buffer A plus 2.0 M NaCl, concentrated by acetone precipitation, and analyzed by SDS-PAGE, and the proteins were transferred to a polyvinylidene difluoride membrane for peptide microsequence analysis.

Determination of Enzyme Activity of hTE—The enzyme activity of hTE was measured spectrophotometrically using Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) as described (37, 38) with 0.125 μg of purified hTE in a volume of 0.25 ml. An hTE radiochemical assay (37, 38) contained [1-^{14}C]palmitoyl CoA (31.4 Ci/mol) in a volume of 0.1 ml and incubations at 25 °C. The free ^{14}C palmitic acid produced was extracted and assayed by liquid scintillation spectrometry.

Glutathione-Agarose Pull-down Assay—Extracts from 1.5 ml of bacteria culture expressing GST-Nef fusion proteins were prepared by sonication in 1 ml of PBS, 50 mM EDTA, 10% glycerol, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. Purified hTE (50–100 μg) was added to these crude bacterial lysates along with 10–20 μl of glutathione-agarose beads. Binding was carried out at 4 °C for 1 h. The beads were spun down and washed with the binding buffer containing 0.1% Triton X-100 and 1 mM EDTA. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE/Coomassie Blue staining of the gel.

Cell Lines, HIV Preparation, and Flow Cytofluorimetry Analysis—The human lymphoblastoid cell line 721.221 expressing CD4 and HLA-A2 (17) and Bosc cells (39) are as described. Human embryonic kidney 293 cells and Bosc cells were transfected with various DNAs by the calcium phosphate precipitation method (39).

HIV was generated from transfected 293 cells and HIV infection of target cells was done as described (40). Wild-type and various Nef mutants were cloned into the NL-PI vector derived from the molecular clone NL4–3 that carries the full complement of HIV genes as well as encoding for the placental alkaline phosphatase (PLAP) reporter gene (40). Single-round infectivity assays were performed using CD4-positive HeLa cells containing an HIV-LTR-βGal reporter (MAGI cells) (41).

Jurkat T-cells and CD4 positive 721.221 cells expressing HLA-A2 were infected with HIV-1 reporter virions encoding for PLAP. 2 days post-infection, the cells were stained for CD4, MHC class I, or PLAP expression followed by cytofluorimetry on a Becton Dickinson FACSCAN as described (17, 40). Down-regulation of CD4 in 293 cells was monitored after cotransfection of plasmids encoding for CD4 and various Nef mutants into 293 cells. CD4 expression levels were measured 2 days post-transfection by FACS. Nef expression levels were monitored by Western blot analysis.

Immunofluorescence Microscopy—Nonreplication competent murine ecotropic retroviruses carrying the Nef-GFP fusion gene or Flu-tagged hTE were made using the retrovirus packaging cell line Bosc (39). Nef-GFP and Flu-hTE retroviruses were used either alone or together to (co)infect mouse NIH-3T3 cells. Infected 3T3 cells were grown on coverslips and stained 2 days post-infection. Cells were fixed in paraformaldehyde/PBS, washed in PBS buffer, quenched in 50 mM NH_4Cl/PBS, and permeabilized in PBS containing 0.1% Triton X-100 and 1 mg/ml BSA. Cells were incubated with anti-Flu tag antibodies, washed three times, and stained with a Texas Red-conjugated secondary antibody. After three washes in PBS, cells were mounted on microscope slides in 100 mM Tris-HCl, pH 8.5, 100 mg/ml Mowiol, 25 mg/ml 1,4-diazabicyclo[2.2.2.]octane, and 25% glycerol and examined under a epifluorescence microscope attached to a CCD camera or to a confocal microscope. No immunofluorescence staining was observed when secondary antibodies were used without the primary antibody or with an irrelevant primary antibody.

RESULTS

Nef Associates with a Human Thioesterase—When cell lysates from human Jurkat T-cells were applied to a GST-Nef affinity column (NL4–3 Nef allele), a 35-kDa protein bound to the column, but it did not bind to a control column containing the GST protein alone (data not shown). Because certain functions of HIV-Nef such as CD4 down-regulation can be species-independent (13, 42), we tested whether a 35-kDa protein might also be found in a T-cell extract derived from calf thymus. For this experiment we used an affinity column of Nef alone covalently attached to Affi-Gel beads. Once again, only a 35-kDa protein was seen to specifically associate with the Nef affinity column but not with the control column (Fig. 1*A*, *lanes 1* and *3*). The protein seen at 27 kDa in *lane 1* is Nef that was noncovalently attached to the column and came off when the beads alone, which had not been exposed to thymus extract, were boiled in SDS loading buffer (Fig. 1*A*, *lanes 4* and *5*). Similarly, the proteins seen at 55 kDa in *lane 1* were not derived from the T-cell extract because they were present when the beads alone were boiled in loading buffer (Fig. 1*A*, *lanes 4* and *5*), and they most likely represent dimerized Nef or residual GST-Nef from which the Nef was derived. The association of the 35-kDa protein (p35) with Nef was independent of the presence of the PxxP motif in Nef because a mutant Nef lacking the motif (P72A, P75A) (24), still bound to p35 (Fig. 1*A*, *lane 2*). Therefore, the 35-kDa protein probably is not binding to Nef through a SH3 domain interaction.

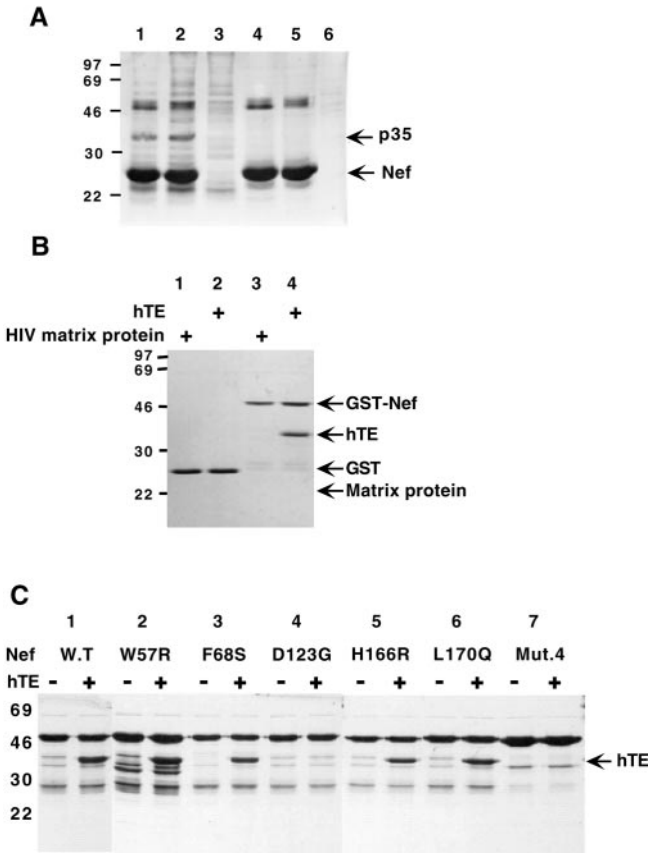

FIG. 1. *A*, a 35-kDa protein specifically interacts with HIV NL4–3 Nef. Calf thymus extract was applied to an affinity column containing wild-type Nef-AffiGel (*lane 1*), P72A + P75A mutant Nef-AffiGel (*lane 2*), or AffiGel column alone (*lane 3*). After extensive washing, the gel material was boiled in SDS loading buffer, proteins bound to the column were separated by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. *Lanes 4–6* are beads corresponding to *lanes 1–3*, respectively, except that these beads were never exposed to calf thymus extract. *B*, association of Nef with hTE *in vitro*. Purified hTE (*lanes 2* and *4*) or HIV matrix protein (*lanes 1* and *3*) were incubated with lysates from bacterial cells expressing either GST (*lanes 1* and *2*) or Nef-GST fusion protein (*lanes 3* and *4*) in the presence of glutathione-agarose beads. After extensive washing, proteins bound to the glutathione-agarose beads were separated by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. *C*, ability of various Nef mutants to interact with hTE *in vitro*. Purified hTE was either absent or added to lysates from bacterial cells expressing GST fusions of various Nef mutants in the presence of glutathione-agarose beads (the absence or presence of added hTE is indicated as − or + in the hTE row above the gel). Bound proteins were analyzed by SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. The ability of the 35-kDa hTE to bind to the Nef mutant is indicated by the presence of an additional band at 35 kDa in the lane that contains added hTE (*hTE +*).

The 35-kDa protein was purified from the calf thymus extract and subjected to peptide microsequence analysis. All seven peptide sequences obtained by peptide microsequence analysis (data not shown) were found in a single human expressed sequence tag data base clone. Using this information, we cloned the cDNA for a protein that is 42% identical to a bacterial protein called thioesterase II. This protein termed hTE was overexpressed in *E. coli* with a histidine tag at the N terminus of the protein. That the recombinant hTE and Nef could interact directly was then demonstrated *in vitro*. Purified His-tagged hTE or His-tagged HIV matrix protein (a control protein) were incubated with a bacterial cell lysate from cells expressing either GST-Nef or GST. Proteins present in the glutathione-agarose bound fraction were separated by SDS-PAGE. As shown in the Fig. 1*B*, GST-Nef was able to bind hTE (*lane 4*) but not the HIV matrix protein (*lane 3*), whereas GST alone was unable to bind hTE (*lane 2*) or the HIV matrix protein (*lane 1*). Based on the intensity of staining with Coomassie blue, Nef and hTE appear to interact in an approximately stoichiometric ratio.

We also investigated whether Nef can interact with the *E. coli* TE II homolog or rat mammary gland thioesterase II (43) (rat TEII belongs to a class of thioesterases that do not share a sequence similarity to hTE). However, we found that neither of these thioesterases could interact with HIV Nef in the GST pull-down assay (data not shown). Therefore, the amino acid residues in hTE that are not conserved in the *E. coli* homolog are important in the interaction with HIV Nef.

Enzymatic Properties of hTE—The homolog of hTE from *E. coli* hydrolyzes acyl-CoAs of various chain lengths (35). Therefore, hTE was assessed for its ability to utilize various acyl-CoAs as substrates. Enzyme activity of hTE was measured either by a spectrophotometric assay or a radiochemical assay (37, 38). The hTE protein hydrolyzed acyl-CoA substrates of chain lengths C8, C10, C12, C14, and C16. The V_{max} values for these substrates are 9, 6, 5, 8, and 8 μmol/min/mg respectively. The turnover number (k_{cat}) of hTE with C10-CoA as a substrate is about 12-fold lower than that of *E. coli* thioesterase II. The pH optimum for the enzyme was between pH 8.0 and 8.2, similar to that of the *E. coli* enzyme. Kinetic analysis of the enzyme revealed that hTE, like its *E. coli* counterpart, works on a broad range of acyl chain length acyl-CoAs, but K_m values tend to increase with decreasing chain length. The K_m value for C8, C10, C12, C14, and C16 acyl-CoA substrates is 82, 20, 4, 4, and 2 μM, respectively. Therefore, the specificity of the enzyme, as measured by V_{max}/K_m, shows a preference for longer chain fatty acids. Incubation of the purified hTE with a 7-fold excess of GST-Nef, conditions known to favor the hTE-Nef interaction, had no effect on the enzyme activity either under substrate-limiting ($s < K_m$) or saturating ($s > K_m$) conditions when assayed using decanoyl-CoA as a substrate (data not shown).

Analysis of Subunit Structure of hTE—Earlier studies had indicated that *E. coli* thioesterase II exists as a tetramer (35). Therefore, the subunit structure of hTE was investigated by gel filtration high pressure liquid chromatography using a Sigmachrom GFC-1300 (300 × 7.5 mm) column. We found that the oligomeric status of hTE is highly dependent on protein concentration. At 3 mg/ml hTE ran as a tetramer on a gel filtration column with no sign of any material corresponding to either a dimer or monomer of hTE. However, upon dilution to 0.5 mg/ml, hTE ran as a dimer. Both the tetrameric and dimeric forms of hTE exhibited similar enzymatic activity (data not shown).

The hTE protein contains eight cysteine residues. However, because β-mercaptoethanol treated and untreated hTE both ran as a single band corresponding to a molecular mass of 35 kDa when subjected to SDS-PAGE, it is unlikely that disulfide bridges contribute to the polymerization of the protein (data not shown).

Identification of hTE Binding Site on Nef—A previous report of Nef interaction with hTE identified a mutant, called Mut.4, that did not interact with hTE (33). This Nef mutant contains 5 point mutations from the original Nef-LAI wild-type sequence: W57R, F68S, D123G, H166R, and L170Q. To identify residue(s) that are critical for interaction with hTE, we mutated the above residues in Nef allele NL4–3 individually and tested the resulting mutant Nef proteins for their ability to interact with hTE in a Gst-Nef pull-down assay (Fig. 1*C*). Except for the Nef protein carrying the mutation, D123G, all the other single Nef mutants exhibited normal interaction with hTE. We also did not detect an interaction between D123G Nef and hTE in a yeast two-hybrid screen (data not shown). Because wild-type and D123G Nef show similar CD spectra, tem-

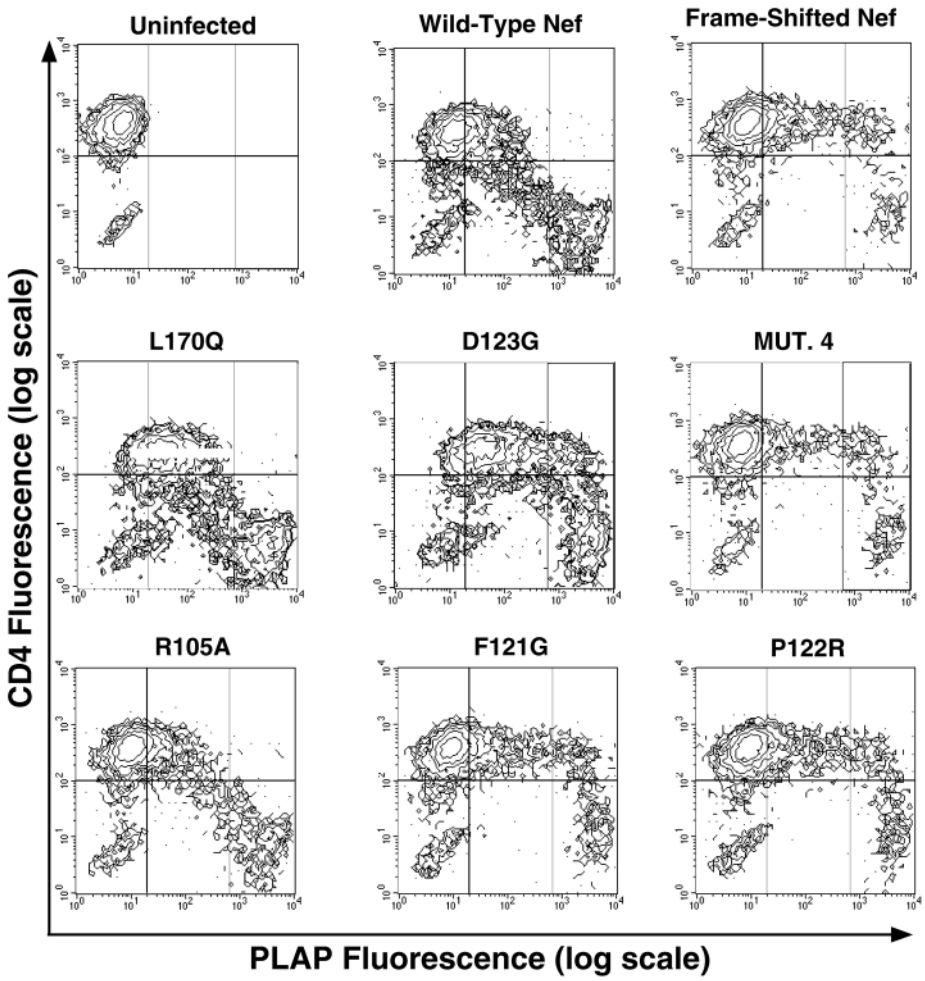

FIG. 2. **Flow cytometric analysis of the ability of Nef mutants to induce CD4 down-regulation.** Wild-type and various Nef mutants were cloned into the NL-PI vector derived from the HIV molecular clone NL4–3 that carries the PLAP reporter gene. Human Jurkat T-cells were infected with HIV-1 reporter virions and 2 days later were stained for CD4 (*y* axis) and PLAP (*x* axis).

perature melt CD curves, and the ability to interact with the Hck SH3 domain in a GST pull-down assay (data not shown), these results suggest that this mutation does not cause a global disruption in Nef structure. Moreover, *in vivo* the D123G Nef mutant retains some of its Nef-associated activities (see below).

The mutant D123G Nef and other Nef mutants were then cloned back into HIV strain NL4–3 (NL-PI) carrying the PLAP reporter gene. PLAP is a cell surface protein, and PLAP expression allows us to follow HIV infection of cells by flow cytometry (40). The ability of these various mutants to down-regulate CD4 was assessed in HIV-infected Jurkat cells. NL-PI carrying wild-type Nef alleles down-regulates CD4 rapidly in this assay. Therefore, there appears to be a direct inverse relation between PLAP expression and CD4 levels in Jurkat cells (Fig. 2). However, in NL-PI carrying a frameshift in Nef, there is little CD4 down-regulation until there are high levels of PLAP expression. CD4 down-regulation in these cells is due to the synthesis of two late HIV genes, Vpu and Env (40). F68S Nef, H166R Nef, and L170Q Nef proteins that interact with hTE were able to induce CD4 down-regulation similar to the wild-type protein (Fig. 2 and Table I). However, neither D123G Nef (Fig. 2) nor W57R Nef (Table I) induced CD4 down-regulation either within the context of HIV infection of T-cells or in cotransfection assays of CD4 and Nef into human 293 cells (data not shown). The Trp^{57} residue has been speculated to play a role in direct binding to CD4 (44); however, mutation of another Nef residue, E59A, also speculated to interact with CD4, had no effect on CD4 down-regulation (data not shown). Although there was a small variability in the expression level of these various mutants relative to the wild-type Nef protein

TABLE I
Ability of mutant Nef alleles to induce CD4 down-regulation and bind to hTE

Ability of Nef alleles to down-regulate CD4 was determined either by cotransfection of 293 cells with plasmids encoding for CD4 and various mutant Nef alleles or by infection of Jurkat T cells with HIV-1 virions encoding for PLAP and various mutant Nef alleles. Down-regulation of CD4 was monitored by flow cytometry as described under "Experimental Procedures." Association of mutant Nef alleles with hTE was followed by GST-Nef pull-down assay as discussed under "Experimental Procedures." The amino acid numbering for SF2 Nef is based on the corresponding residue in NL4–3 Nef to facilitate comparisons.

Nef allele	CD4 downregulation	Interaction with hTE
NL4–3 wild type	+	+
F68S	+	+
R105A	+	+
R106A	+	+
H166R	+	+
L170Q	+	+
P78S	+	+
Mut.4	–	–
D123G	–	–
D108A	–	–
L112D	–	–
F121G	–	–
P122R	–	–
D123V	–	–
L76P	–	+
W57R	–	+
E59A	+	+
D108E	+	–
SF2 wild type	+	–
E108D	+	+
E108A	+	Not determined
D123A	–	Not determined
SIV Mac239	+	–

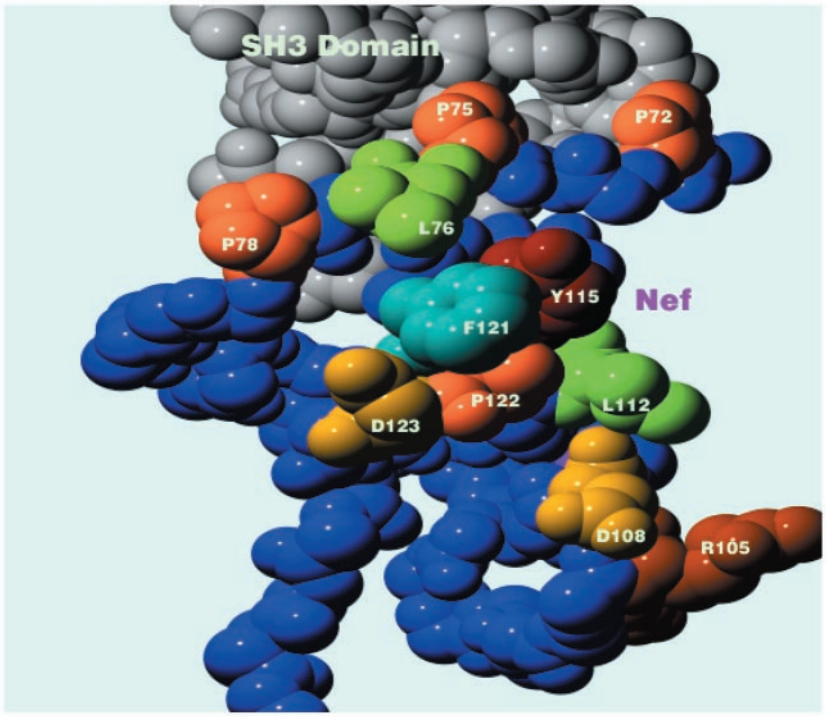

FIG. 3. **Structure of Nef-SH3 complex.** NL4–3 Nef residues 71–130 and SH3 domain residues 85–141 were used to generate the space filling model of Nef-SH3 complex according to Lee *et al.* (46).

(at most 2-fold), we saw no correlation between the expression level of the Nef mutants and their ability to down-regulate CD4.

In the NMR and crystal structures of Nef (44–46), Asp^{123} lies in close proximity to Pro^{72} and Pro^{75} of the SH3 binding P*XX*P motif (Fig. 3). Mutation of Pro^{72} and Pro^{75} affected viral infectivity and MHC class I down-regulation but did not significantly affect CD4 down-regulation (23, 24, 47, 48) or interaction with hTE (Fig. 1). To further characterize this region, we made additional mutations and assessed the mutants for their ability to down-regulate CD4. Because we were concerned that mutations in this region might destabilize Nef, to maximize our chances of obtaining stable Nef mutants we choose eight residues that cluster next to Asp^{123} in the crystal structure of Nef (Fig. 3; Leu^{76}, Pro^{78}, Asp^{108}, Leu^{112}, Tyr^{115}, Phe^{121}, Pro^{122}, and Asp^{123}), and for each of these residues, we individually randomized the codon for that residue and cloned the mutant library into the pGEX2T vector. Ten bacterial colonies were then isolated for each residue mutated and tested for their ability to interact with hTE in the GST pull-down assay. Analysis of these mutants told a consistent story. Mutation of Leu^{76} and Pro^{78} (and also R105A and R106A) had little effect on hTE binding, whereas mutations at Asp^{108}, Leu^{112}, Phe^{121}, Pro^{122}, and Asp^{123} abrogated Nef association with hTE (Fig. 4). Mutations at Tyr^{115} tended to destabilize the protein as judged by protein expression levels. Thirty-two of these mutations were then sequenced, and although the mutagenesis was not entirely random, it had introduced variability at each residue (Fig. 4).

Of the above 80 Nef mutants we chose representatives that appeared to be stable, based on expression levels in *E. coli,* and cloned them into the HIV NL-PI vector and tested them for their ability to induce CD4 down-regulation. Mutations that abolished the interaction with hTE abrogated Nef-induced CD4 down-regulation, whereas mutations that did not affect hTE binding generally had no influence on CD4 down-regulation (Fig. 2 and Table I). Based on the structure of Nef (44–46), residues that affect hTE binding lie together on a patch on the surface of Nef, whereas those residues that do not affect hTE binding lie at the periphery of this patch.

Some of these mutants were also tested for their ability to promote MHC class I down-regulation (Fig. 5). In general, it appears that although this region on Nef is important also for class I down-regulation, the CD4 and class I down-regulation regions on Nef are overlapping but distinct regions. This is in agreement with previous reports that Pro^{72} and Pro^{75} in this region are more important for class I down-regulation than CD4 down-regulation (23, 24, 47, 48). The ability of some of these mutants to down-regulate class I but not CD4 (*e.g.* L112D; Fig. 5) further supports our contention that these mutations do not globally disrupt Nef structure. In addition, although wild-type NL4–3 virions are approximately 10-fold more infectious in a single-cycle infectivity assay (41) than NL4–3 virions carrying a frameshift in Nef, the Nef/hTE association mutants were 5-fold (D123G) to 8-fold (D108A) more infectious than frameshifted Nef.[2]

Role of the N Terminus of Nef in hTE Binding—The N-terminal region of Nef functions in both CD4 and class I down-regulation (25, 47). However, in the NMR structure of Nef this region was not well ordered (44, 45). Deletion analysis of Nef suggested that this region might influence hTE binding (data not shown). To further demonstrate the role of the Nef N terminus in hTE binding, residues 1–35 of NL4–3 Nef were attached to GST (GST-Nef 1–35), and its ability to associate with hTE in the GST pull-down assay was tested. As shown in Fig. 6*A*, neither hTE alone nor Nef 36–206 alone (this contains residues 36–206 of NL4–3 Nef and is not part of a GST fusion) interacted with GST-Nef 1–35 (*lanes 4* and *5*). However, when Nef 36–206 and hTE were added together to GST-Nef 1–35, both proteins tightly bound the N-terminal region of Nef (*lane 6*) but not to GST alone (*lane 2*). The simplest explanation of this experiment is that either hTE binds to both the core domain of Nef (36–206) as well as its N terminus (1–35) or that

[2] G. B. Cohen, V. S. Rangan, B. K. Chen, S. Smith, and D. Baltimore, unpublished results.

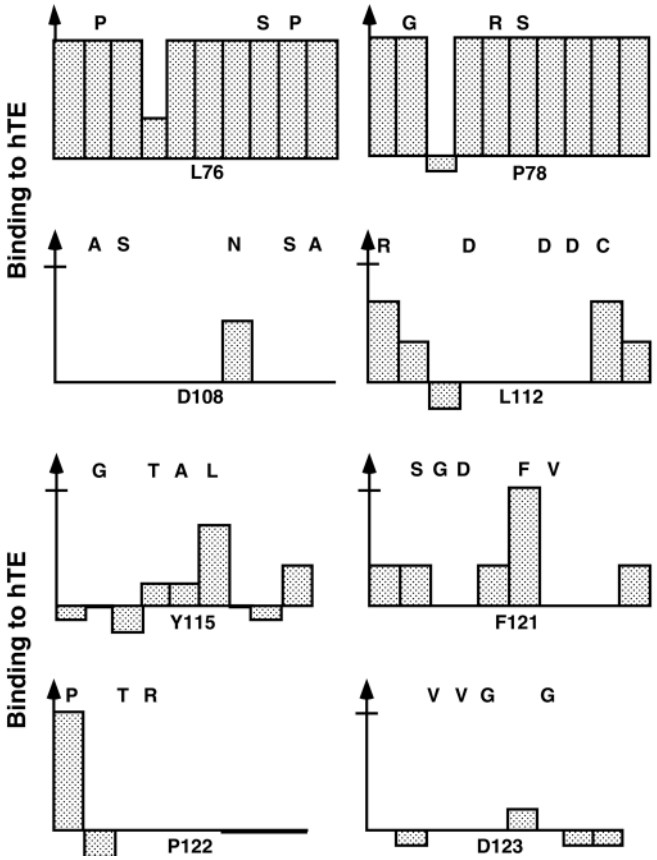

FIG. 4. **Random mutagenesis of Nef at defined positions.** A random Nef mutant library at defined residues was created (see "Experimental Procedures" for details). For each residue mutated, 10 bacterial colonies were picked and tested in the GST pull-down assay for hTE association. The results of this analysis are summarized in this figure. Strong hTE binding is depicted by the height of the *bar* on the *y* axis. Nef mutants that were poorly expressed and did not bind to hTE are depicted by a negative height on the *y* axis. Of the 80 mutants tested, 32 were sequenced to determine the residue introduced by the random mutagenesis. For the mutants that were sequenced, the amino acid found at that residue is indicated in the graph by the single-letter amino acid code above the *bar graph*.

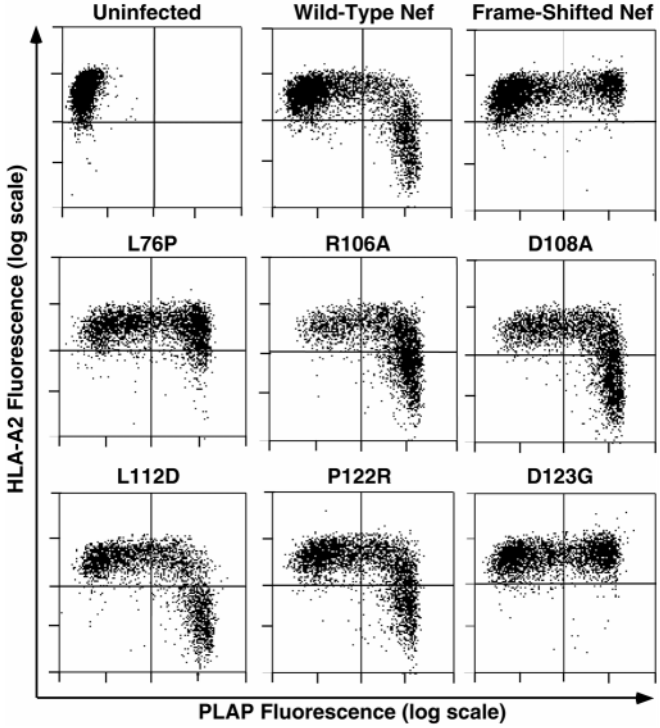

FIG. 5. **MHC class I down-regulation by Nef mutants.** Human CD4-positive 721.221 cells that expressed HLA-A2 on the cell surface were infected with HIV-1 NL4–3 PLAP reporter virus and 2 days later were stained for HLA-A2 surface expression (*y* axis) and PLAP (*x* axis) as described previously (17).

the interaction of hTE with the core region of Nef increases the avidity of the core region of Nef for the Nef N terminus. This might suggest that upon hTE binding the N-terminal region of Nef assumes a stable structure.

Nef Can Bind Multiple Proteins at the Same Time—Because the SH3 binding site and hTE binding site lie in close proximity on Nef, we tested whether Nef is able to associate with an SH3 domain and hTE at the same time. Lysates from bacteria expressing a GST fusion with the SH3 domain from Hck (GST-Hck-SH3) were incubated with Nef and hTE, either separately or together, in the presence of glutathione-agarose beads. After washing the beads were boiled in loading buffer, and bound proteins were analyzed by SDS-PAGE. As shown in the Fig. 6*B*, hTE by itself was unable to bind to the SH3 domain (*lane 2*), whereas in the presence of Nef it could co-associate with the SH3 domain (*lane 4*). In contrast, the D123G Nef mutant, although it still binds to the SH3 domain (*lane 5*), lacks the ability to bind hTE (*lane 6*). Nef could therefore function *in vivo* as an adapter protein, binding multiple cell signaling proteins at the same time.

HIV Nef Proteins Differ in Their Affinity for hTE—The association of Nef with a cellular serine/threonine and enhancement of viral infectivity by Nef varies with different Nef alleles (49). Therefore, three other HIV-1 Nef alleles (LAI, SF2, and BO Nef; BO is a primary Nef allele isolate provided by Dr. David Ho) were tested for hTE binding using the yeast two-hybrid and GST pull-down systems. With the exception of SF2 Nef, the two other Nef proteins bound tightly to hTE (data not shown; see also Nef alleles that bound hTE in previous studies (33)). Overall, NL4–3 Nef and SF2 Nef, differ by 30 amino acid residues, and SF2 Nef does down-regulate CD4 in human cell lines (42). To localize the residues responsible for hTE association, the following SF2/NL4–3 chimeric proteins linked to GST were engineered; NL4–3(1–70)/SF2(71–206), NL4–3(1–125)/SF2(126–206), and NL4–3(1–173)/SF2(174–206) (amino acid residue numbers refer to the corresponding residue in NL4–3 Nef) and tested for their ability to interact with hTE. Only NL4–3(1–70)/SF2(71–206) did not interact with hTE (data not shown). These results indicate that the region between amino acid residues 70–125 in NL4–3 Nef are probably required for association with hTE. Because Asp^{108}, previously identified in the hTE binding site of NL4–3 Nef, is a glutamic acid in SF2 Nef, we swapped these two residues between NL4–3 and SF2 Nef. As shown in Fig. 7, D108E NL4–3 Nef did not associate with hTE, whereas E108D SF2 Nef showed potent hTE binding activity (in SF2 Nef the homologous residue is residue 112 but to simplify comparisons we use the NL4–3 numbering system here). These results indicate that in SF2 Nef, the presence of Glu instead of Asp at position 108 is responsible for the lack of interaction with hTE. Although many HIV B-strains (frequent in Western Europe and North America) contain an Asp at this position and are predicted to bind to hTE, many other strains of HIV-1 as well as HIV-2 and SIV Nef contain the Glu and will not associate with hTE. As predicted, the one SIV Nef allele tested, Mac239, which contains a Glu at this residue, did not bind to hTE (data not shown).

Nef Colocalizes with hTE in the Peroxisomes—Northern blot analysis revealed that the hTE gene was expressed in all tissues examined including peripheral blood leukocytes (Ref. 33

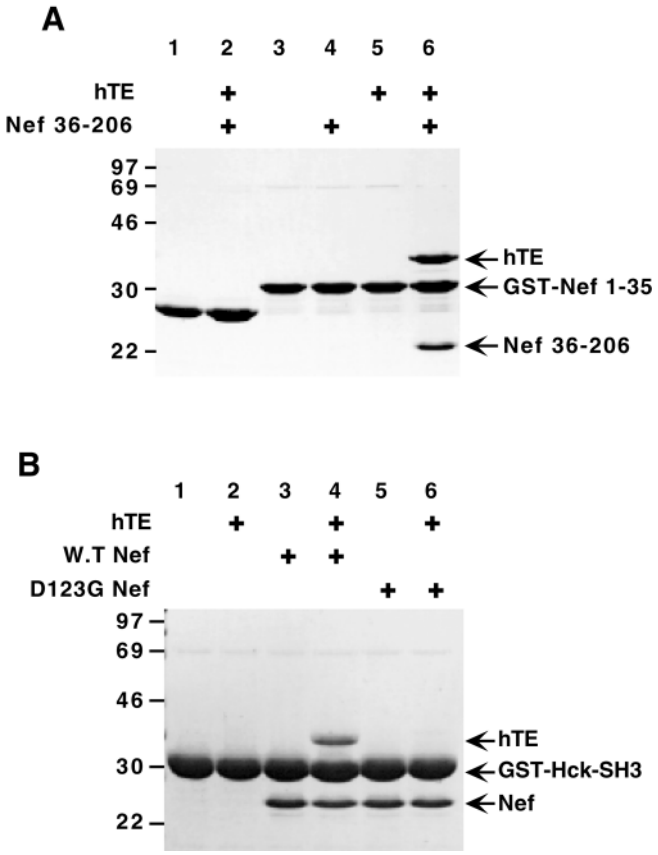

FIG. 6. *A*, role of the N-terminal region of Nef in hTE binding. NL4–3 Nef containing residues 36–206 and hTE were incubated with lysates from bacteria expressing GST fusion of NL4–3 Nef carrying Nef residues 1–35 in the presence of glutathione-agarose beads. Proteins bound to the gel material were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. *Lane 1*, GST alone; *lane 2*, GST in the presence of hTE and Nef 36–206 (Nef 36–206 is not a GST fusion protein); *lane 3*, GST-Nef 1–35 alone; *lanes 4* and *5*, GST-Nef 1–35 in the presence of Nef 36–206 and hTE, respectively; *lane 6*, GST-Nef 1–35 in the presence of both hTE and Nef 36–206. *B*, Nef can bind multiple proteins at the same time. Lysates from bacteria expressing a GST fusion protein with the SH3 domain from Hck (GST-Hck-SH3) were incubated with purified wild-type Nef, D123G Nef, or hTE in the presence of glutathione-agarose beads. Bound proteins were analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. *Lanes 1–6* contain GST-Hck-SH3 and additions described as follows: *lane 1*, no addition; *lanes 2*, hTE; *lane 3*, wild-type Nef (not a GST fusion Nef); *lane 4*, both hTE and wild-type Nef; *lane 5*, D123G Nef; *lane 6*, both hTE and D123G Nef.

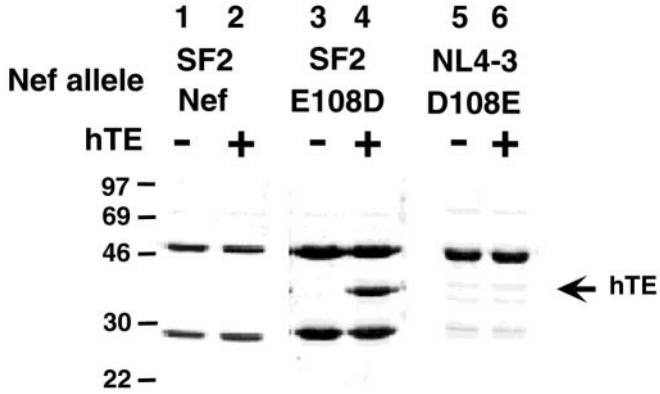

FIG. 7. **HIV Nef proteins differ in their affinity for hTE.** Bacterial cell lysates containing either GST fusion of wild-type SF2 Nef (*lanes 1* and *2*), E108D SF2 Nef (*lanes 3* and *4*), or D108E NL43 Nef (*lanes 5* and *6*) were incubated either in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4*, and *6*) of purified hTE.

and data not shown). We then wished to determine the subcellular localization of hTE and whether Nef and hTE colocalize *in vivo*. The subcellular location of Nef and hTE was studied using Nef tagged at the C terminus with GFP and hTE tagged at the N terminus with Flu peptide. In the cells that expressed hTE, a highly punctate pattern of staining, indicative of an association with an intracellular organelle, was observed (Fig. 8*A*). Because hTE, but not its *E. coli* counterpart, contains a C-terminal tripeptide serine-lysine-leucine (SKL) peroxisomal targeting motif (50), these organelles seemed likely to be peroxisomes. Deletion of the C-terminal SKL peroxisomal signal sequence in hTE resulted in a diffuse cellular staining pattern (Fig. 8*A*). Furthermore, although GFP alone displays a diffuse staining pattern in cells (Fig. 8*B*), upon addition of the SKL signal sequence at its C-terminal end (GFP-SKL), it is targeted to peroxisomes (51). In cells coinfected with GFP-SKL and Flu-tagged hTE, there was a striking correspondence in the punctate cellular staining pattern (Fig. 8*C*), suggesting that hTE is predominantly a peroxisomal protein. This is in agreement with a recent report that the endogenous hTE is a peroxisomal protein (52).

In NIH 3T3 cells infected with a retrovirus encoding the Nef NL4–3-GFP fusion protein, we observed a rather diffuse distribution of Nef-GFP throughout the cell and a more concentrated fluorescence pattern at the plasma membrane and in the perinuclear region (Fig. 8*D*) consistent with earlier reports (28, 48). In contrast, in many of the cells coexpressing both Nef-GFP and hTE, Nef-GFP displayed a punctate pattern of distribution that largely colocalized with hTE in the peroxisomes (Fig. 8, *E* and *F*). This colocalization was seen in approximately 10–20% of the cells that coexpressed Nef and hTE. Colocalization of Nef to peroxisomes in the presence of hTE was abolished upon removal of the SKL signal sequence from hTE; however, the two proteins still appeared to colocalize in other parts of the cell (data not shown).

Similar colocalization experiments were conducted with the Nef alleles that we previously found do not bind to hTE *in vitro*. Neither SF2 Nef-GFP (Fig. 8*H*) nor SIV-Mac239-Nef-GFP (data not shown) appeared to colocalize with hTE when coexpressed in NIH 3T3 cells. The punctate staining seen for both SF2 (data not shown) and SIV Mac 239 Nef-GFP (Fig. 8*G*) alleles in the absence of hTE has been previously described and results from Nef colocalization with clathrin-coated vesicles (28). Surprisingly, we found that D123G NL4–3 Nef-GFP, which like the wild-type NL4–3 Nef has a much diminished affinity to localize to clathrin coated vesicles (and does not show an obvious punctate staining pattern in the absence of hTE), colocalized with hTE upon coinfection into NIH 3T3 cells to an extent similar to that of the wild-type protein (Fig. 8*I*). This is in marked contrast to the complete inability of D123G NL4–3 Nef to bind hTE in either the GST pull-down assay (Fig. 1*C*) or yeast two-hybrid assays.[2] This observation suggests that *in vivo* other proteins may help bring Nef and hTE together. Alternatively, in the Nef-GFP fusion, the N-terminal of Nef is free and probably is myristoylated (in both of the *in vitro* assays Nef is fused at its N terminus and would not be myristoylated). Because hTE has a fatty acid binding site that it uses to bind to substrates like palmitoyl-CoA, the myristoylated Nef N terminus could bind to this site and facilitate the interaction of Nef with hTE, *in vivo*, in the absence of binding to the core region of Nef.

DISCUSSION

Characterization of hTE—Using a Nef affinity column, we have isolated and identified from T-cell lysates a 35-kDa Nef-interacting protein, hTE. hTE had previously been identified as

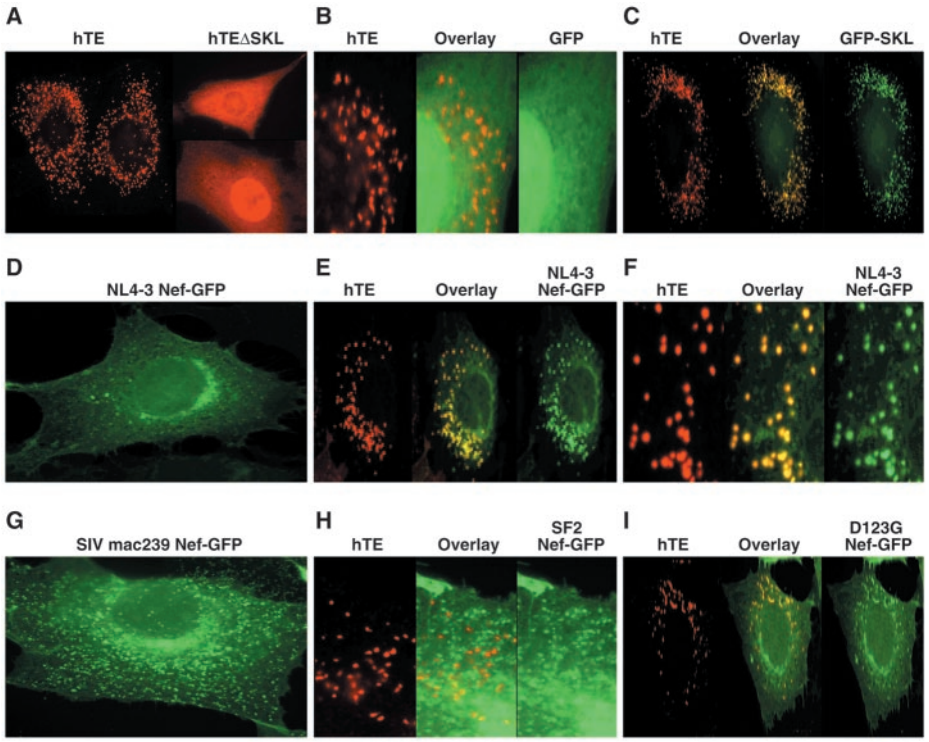

FIG. 8. **Nef colocalizes with hTE in the peroxisomes.** Mouse 3T3 cells were infected with retroviruses encoding Flu-tagged hTE and various Nef-GFP fusion proteins. *A*, wild-type hTE and hTE lacking the C-terminal SKL sequence; *B*, coinfection with hTE and GFP; *C*, coinfection with hTE and GFP-SKL; *D*, NL4–3 Nef-GFP alone; *E*, coinfection with hTE and NL4–3 Nef-GFP; *F*, coinfection with hTE and NL4–3 Nef-GFP, close-up view; *G*, wild-type SIVmac239Nef-GFP alone; *H*, coinfection with hTE and SF2 Nef-GFP; *I*, coinfection with hTE and D123G NL4–3 Nef-GFP. The distribution of Flu-tagged hTE in cells was examined by anti-Flu antibodies followed by fluorescent Texas Red-conjugated secondary antibodies. *Overlay* in *C*, *E*, *F*, *H*, and *I* represents the images of the localization of hTE and Nef-GFP overlaid. Overlap of *red* (hTE) and *green* (GFP) results in *yellow color*.

a Nef-interacting protein in yeast two-hybrid screens that used Nef as the bait (33, 34). hTE exhibits a similar substrate specificity and pH profile as its *E. coli* homolog. Binding of Nef to hTE had no effect on thioesterase activity. We suspect that previous reports that Nef influences the kinetic properties of hTE (34) or that the preferred substrate for hTE are short chain fatty acids (33) may be due to the tendency of the longer chain fatty acids to form micelles at relatively low concentrations. These negatively charged micelles may disrupt the native conformation of hTE and complicate the kinetic analysis (53) because we have found that palmitoyl-CoA, in concentrations in excess of its K_m, inhibits hTE activity.

Cellular Function of hTE—Because hTE and its *E. coli* counterpart hydrolyze the thioesterase bond of many long chain acyl-CoA substrates, this has led to the suggestion that it may be involved in fatty acid oxidation and lipid metabolism. Consistent with a general housekeeping role for hTE, the enzyme has been found in all organisms and all tissues examined so far. Our finding, and a recent report (52), that hTE localizes to peroxisomes would seem to support this speculation. Thioesterases in peroxisomes are presumed to regulate the local concentrations of acyl-CoA species and thus the extent of β-oxidation of fatty acids (54). Yet at least in bacteria, overexpression or deletion of TEII led to no detectable change in fatty acid levels (35). Therefore, the ability of a protein to catalyze the hydrolysis of acyl-CoA substrates *in vitro* does not in itself provide sufficient evidence to conclude that acyl-CoA is the likely substrate.

An attractive alternative role for a thioesterase might be in the lipid modification of proteins because palmitoylation of proteins occurs through a thioester linkage to cysteine residues. Many cell-signaling molecules such as Ras and Src family members, seven transmembrane receptors, and CD4 itself are palmitoylated. However, we have not been able to detect any catalytic activity of this enzyme to remove palmitate from protein substrates. Furthermore, mutation of the two palmitoylation sites in CD4 (55) neither influenced the ability of wild-type Nef to down-regulate CD4 nor increased the activity of the D123G mutant toward the palmitoyl-free CD4.[2]

Nef/hTE Interactions—Our data show that residues 108, 112, 121, 122, and 123 in Nef are critical for binding to hTE. Based on the structure of Nef (44–46), these residues define a surface on Nef essential for CD4 down-regulation. This region may also play a role in Nef dimerization, and the dimer of Nef is speculated to be the active form of Nef *in vivo*.[3] The hTE binding site on Nef also lies in close proximity to the SH3 domain-binding site of Nef. However, hTE and SH3 binding do not appear to overlap because Nef was able to bind both the Hck SH3 domain and hTE at the same time (Fig. 6*B*). These results suggest that *in vivo* Nef may function as an adaptor protein linking HIV into multiple cell signaling pathways simultaneously.

We have also demonstrated that in mouse 3T3 cells, expression of hTE promotes the relocalization of Nef to peroxisomes. This Nef/hTE colocalization is dependent upon the presence of C-terminal peroxisomal targeting sequence, SKL, in hTE. In the absence of this signal, Nef and hTE colocalization occurs predominately at the plasma membrane (data not shown). Whether Nef has any need to be localized to peroxisomes is not clear. When both Nef and hTE are overexpressed, hTE may recruit Nef to peroxisomes, but when Nef is present in excess over hTE-infected as in HIV-infected cells, Nef/hTE may colocalize predominantly outside of peroxisomes. In this regard, it is interesting to note that the C-terminal sequence of hTE (C-terminal ESKL) resembles that of a PDZ domain ligand (C-terminal ESxV) (56). PDZ domains play a role in the clustering and subcellular localization of membrane proteins and might suggest that Nef uses the hTE PDZ-like signal to influence the localization of cellular proteins.

Our results also show that although HIV Nef alleles contain many conserved elements they differ in a number of ways, including the affinity for cellular proteins (*e.g.* hTE and a PAK-related kinase (49)), the ability to tolerate the same mutation (*e.g.* residue 108, Table I), and perhaps their subcellular distribution (Fig. 8, *D* and *G*). Whether this reflects true bio-

[3] R. Benarous, personal communication.

logical differences, perhaps reflecting the adaptation of Nef to different cellular environments, remains to be determined. These observations might suggest that different Nef alleles may have evolved slightly different constellations of binding activities much as different HIV envelope proteins use different coreceptors to gain entrance to cells (57) or as HIV and SIV capsid proteins differ in their requirement for cyclophilin (58–61). This could suggest that hTE belongs to a larger family of Nef-interacting proteins or that other Nef-interacting proteins use a domain similar to that found in hTE to interact with Nef, but we have no evidence for this.

The above data suggest a possible role for hTE in Nef-mediated CD4 down-regulation. However, although there is a striking correlation between the ability of NL4–3 Nef to interact with hTE and its ability to down-regulate CD4, we also provide evidence for Nef-induced CD4 down-regulation in the absence of hTE binding. For example, SF2 Nef did not associate with hTE either *in vitro* or by immunofluorescence colocalization. Yet SF2 Nef does down-regulate CD4 in human cell lines (42), and its ability to down-regulate CD4 is also affected by mutations at Asp^{123} (Table I), suggesting that this region of Nef may be needed for CD4 down-regulation in absence of hTE binding. Moreover, even NL4–3 D108E Nef, which has a low affinity for hTE, had near wild-type CD4 down-regulation activity. Lastly, the mutant, D123G NL4–3 Nef, which does not bind to hTE in our *in vitro* assays (yeast two-hybrid or GST pull-down), associated with Nef in NIH 3T3 cells (Fig. 8*I*) yet does not down-regulate CD4 (although it may be argued that the D123G Nef/hTE interaction involves only the N-terminal part of Nef (Fig. 6*A*) and not the core region of Nef and therefore may be unable to promote CD4 down-regulation). Therefore, although the interaction of Nef with hTE appears to be quite strong (Fig. 1*A*) and may involve complex interactions (Fig. 6*A*), the simplest explanation of our data, at this time, is that hTE is not critical for Nef-induced down-regulation.

Whatever its biological function, the Nef/hTE association has been a useful tool to identify a region on Nef critical for CD4 and MHC class I down-regulation. Because mutations in a neighboring region of Nef, Pro^{72} and Pro^{75}, are critical for many of the other functions of Nef (23, 24, 47, 48), these residues define a conserved region on Nef that is a keystone to almost all of the functions of Nef. Therefore, a more detailed understanding of the Nef/hTE interaction could be useful in understanding how a high affinity interaction with Nef is made and in the design of small molecule Nef inhibitors.

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