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 relocation of Nef to peroxisomes. Interaction of Nef and hTE was abolished by point mutations in Nef at residues Asp108, Leu112, Phe121, Pro122, and Asp123. 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) 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 (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 (PXXP) 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 II (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).
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 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 Leu26, Pro78, Asp108, Leu112, Tyr115, Phe121, Pro122, and Asp123**—For each residue mutated we synthesized a random library of all four nucleotide bases. For example, the coding strand primer for mutating NL4–3 Nef at Asp108 contained the sequence CAC TCC AAA AGA CAA NNC TTC TCC GAT CTT GGC ATC (where 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-galactoside. Cells were collected, washed, sonicated, and centrifuged. The supernatant was passed over a nickel-nitrotriacetic 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 thymus were washed in ice-cold PBS, cut into small pieces, and 2 volumes (weight to volume) of ice-cold buffer A (10 mM Glycine, 25 mM Hepes, pH 7.5, 140 mM KCl, 1.3 mM EDTA, 1.0 mM MgCl2, 3.0 mM dithiothreitol) plus 0.01% aprotinin, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM KF, 0.25 mM orthovanadate, and 1 μl 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 a cheesecloth, reprecipitated at 20,000 × g for 45, and then reprecipitated again at 80,000 × g. The supernatant was adjusted to 0.02% azide, held at 4 °C overnight, and resupernatant at 80,000 × g. The supernatant was then preclared 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′-dithio-bis(2-nitrobenzoic acid) (37, 38) with 0.125 μg of purified hTE in a volume of 0.25 ml. An hTE radioactive assay (37, 38) contained [1-14C]palmitoyl CoA (31.4 Ci/mol) in a volume of 0.1 ml and incubations at 25 °C. The free 14C 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, 1% 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 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. Bovine serum albumin proteins were eluted in SDS-PAGE sample buffer and analyzed by SDS-PAGE/Coomassie Blue staining of the gel.

**Cell Lines, HIV Preparation, and Flow Cytometry 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 placential 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 alleles. CD4 expression in 293 cells was 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 (co) to 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, stained with 50 μl NH4C/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 μg/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. 1A, 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. 1A, 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. 1A, 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 target 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. 1A, lane 2). Therefore, the 35-kDa protein probably is not binding to Nef through a SH3 domain interaction.
hTE Binding Site on Nef

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 database clone. Using this information, analysis (data not shown) were found in a single human expressed sequence tag database clone. Using this information, seven peptide sequences obtained by peptide microsequence analysis. All seven peptide sequences obtained by peptide microsequence analysis (data not shown). The 35-kDa protein was purified from the calf thymus extract and subjected to SDS-PAGE. As shown in the Fig. 1B, 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 radioligand 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 $\mu$mol/min/mg respectively. The turnover number (Kcat) 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 $\mu$m, 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 Sigmachrome GFC-1300 (300 x 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 $\beta$-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 hTE interaction with Nef 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. 1C). 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-
per temperature 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, \textit{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 Trp57 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

| 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 | + | - |

| Nef allele | CD4 downregulation | Interaction with hTE |
|------------|-------------------|---------------------|

**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.
(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), Asp123 lies in close proximity to Pro72 and Pro75 of the SH3 binding PXXP motif (Fig. 3). Mutation of Pro72 and Pro75 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 chose eight residues that cluster next to Asp123 in the crystal structure of Nef (Fig. 3; Leu76, Pro78, Asp108, Leu112, Tyr115, Phe121, Pro122, and Asp123), 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 Leu76 and Pro78 (and also R105A and R106A) had little effect on hTE binding, whereas mutations at Asp108, Leu112, Phe121, Pro122, and Asp123 abrogated Nef association with hTE (Fig. 4). Mutations at Tyr115 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. Analyses of these mutants told a consistent story. Mutation of Leu76 and Pro78 (and also R105A and R106A) had little effect on hTE binding, whereas mutations at Asp108, Leu112, Phe121, Pro122, and Asp123 abrogated Nef association with hTE (Fig. 4). Mutations at Tyr115 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).

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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. 6A, 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

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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. 6B, 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 Asp108, 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...
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. 8A). 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. 8A). Furthermore, although GFP alone displays a diffuse staining pattern in cells (Fig. 8B), 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. 8C), 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. 8D) 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. 8E 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. 8H) 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. 8G) 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. 8J). 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. 1C) 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 vitro, 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 SIVmac239 Nef-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. Overlay 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 thioester 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.$^2$

$^2$ 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-medi- cated 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 Asp123 (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 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. 8F) yet does not down-regulate CD4 (although it may be argued that the D123G mutant, D123G NL4–3 Nef, which does not bind to hTE in vitro, is not critical for Nef-induced down-regulation). Therefore, the interaction of Nef with hTE appears to be quite strong (Fig. 1A) and may involve complex interactions (Fig. 6A), 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, Pro27 and Pro30, 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 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 other functions of Nef (23, 24, 47, 48). 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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