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Human Immunodeficiency Virus Type 1 Nef Domains Required for Disruption of Major Histocompatibility Complex Class I Trafficking Are Also Necessary for Coprecipitation of Nef with HLA-A2

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Human immunodeficiency virus type 1 (HIV-1) Nef is a critical protein that is necessary for HIV pathogenesis. Its roles include the disruption of major histocompatibility complex class I (MHC-I) and CD4 trafficking to promote immune evasion and viral spread. Mutational analyses have revealed that separate domains of Nef are required to affect these two molecules. To further elucidate how Nef disrupts MHC-I trafficking in T cells, we examined the role of protein domains that are required for this function (N-terminal alpha helix, polyproline, acidic, and oligomerization domains). We found that each of these regions was required for Nef to disrupt the transport of HLA-A2 to the cell surface and for Nef to coprecipitate with HLA-A2.

To evade cytotoxic T lymphocytes (CTLs), human immunodeficiency virus (HIV) encodes a protein called Nef that inhibits the surface expression of the major histocompatibility complex class I molecules (MHC-I) that are most important for CTL recognition. The exact mechanism Nef uses to disrupt MHC-I cell surface expression is unclear. Nef has been shown to bind the same amino acids in the MHC-I cytoplasmic tail that are required for reduction of MHC-I cell surface expression (31). Nef binding may disrupt MHC-I trafficking by altering transport signals in the MHC-I cytoplasmic tail, by providing new transport signals within Nef, or by promoting the association of MHC-I with a cellular protein.

In addition to affecting MHC-I cell surface expression, Nef accelerates the endocytosis of CD4 (9, 11, 26, 30) through its dileucine motif (4, 8, 10, 12, 19, 20, 23, 27). However, the mechanism used by Nef to affect MHC-I cell surface expression appears to be different. The dileucine motif in Nef that is needed for the acceleration of CD4 endocytosis is dispensable for the disruption of MHC-I trafficking. In addition, regions of Nef that are needed to reduce MHC-I cell surface expression (N-terminal alpha helix, polyproline, and acidic domains) are dispensable for CD4 downmodulation (1, 6, 12, 21, 32).

Multiple studies have identified a subset of Nef domains that are required for its effects on MHC-I (1, 6, 13, 21, 32). These studies have utilized a variety of cell types and expression systems to characterize each mutant. Therefore, to compare the relative importance of each domain, we first established an adenoviral expression system that would provide Nef expression levels in T cells similar to that found during HIV infection. For these studies, we utilized the HIV molecular clone HXB-EP (5, 7), which has a marker gene, PLAP, inserted into the envelope open reading frame. In this construct, Nef is expressed under its normal regulatory elements (Fig. 1A). Primary T cells transduced with HXB-EP displayed a decreased cell surface expression of HLA-A2, which was dependent upon Nef expression (Fig. 1B). Additionally, cells transduced with this HIV molecular clone expressed Nef at levels comparable to unmodified molecular clones derived from primary isolates (Fig. 1C). Therefore, this molecular clone was used as a control to establish an adenoviral multiplicity of infection (MOI) at which CEM T cells could be efficiently transduced to express Nef at levels similar to that found during HIV infection. To determine this MOI, CEM T cells were transduced with a control or Nef-expressing adenovirus at a range of MOIs, stained for either CD4 or HLA-A2, and analyzed by flow cytometry. As shown in Fig. 1D and E, an MOI of 50:1 (based on 293-cell infectivity) yielded Nef expression levels similar to that of HXB-EP and resulted in dramatic effects on MHC-I cell surface expression.

We then used this adenoviral system to examine Nef mutants that are unable to disrupt MHC-I trafficking (Fig. 2A). First, we titrated each mutant Nef adenovirus to achieve expression levels comparable to wild-type Nef (Fig. 2B). Next, each mutant was tested for its capacity to affect HLA-A2 and CD4 surface expression (Fig. 2C and D). In these studies, wild-type Nef reduced HLA-A2 cell surface expression approximately fivefold, while cells expressing Nef mutant $V_{10}E\Delta17$-26, $M_{20}A$, $P_{75/78}A$, or $D_{123}G$ reduced HLA-A2 expression no more than 1.5-fold. Nef mutants $E_{62\text{-}65}Q$ and $P_{72/75}A$ affected HLA-A2 slightly more than the other mutants (approximately twofold) (Fig. 2C and D). A partial effect of mutating the acidic domain and the first polyproline domain of Nef is consistent with prior reports (21, 25, 32). All of these mutants, except $D_{123}G$, were active at CD4 downmodulation (Fig. 2C and D).

There is evidence that Nef primarily disrupts MHC-I cell surface expression in HIV-infected primary T cells by blocking

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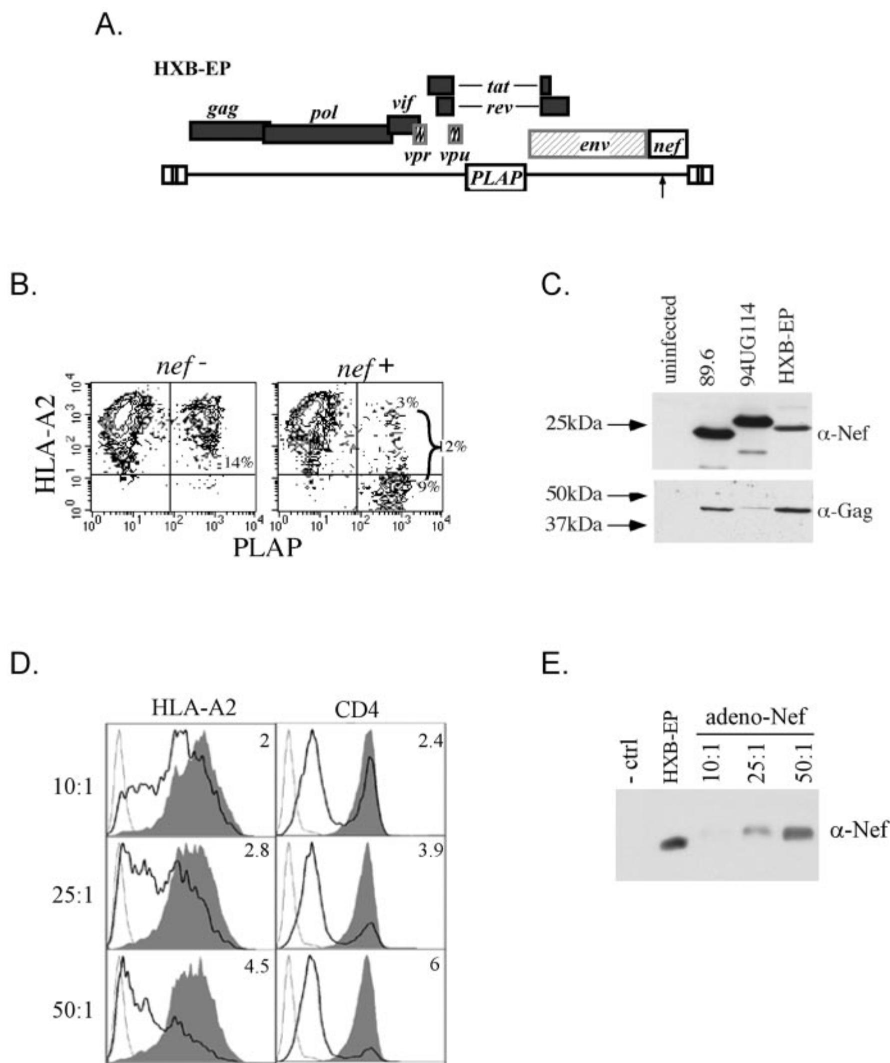

FIG. 1. An adenoviral system that expresses Nef at levels similar to that found during HIV infection. (A) Map of HXB-EP. The marker gene PLAP has been cloned into the envelope open reading frame, and Nef is expressed under its normal regulatory elements. The hatched boxes indicate nonfunctional genes. The arrow indicates the location of the frameshift mutation in Nef that disrupts its expression. (B) Primary T cells transduced with HXB-EP have decreased surface expression of HLA-A2. Primary T cells transduced with HXB-EP that did or did not express Nef were stained for HLA-A2 and PLAP. Cells falling to the right of the vertical line are PLAP positive (infected), and cells falling below the horizontal line are negative for HLA-A2. The percentage of infected cells is indicated, as well as the percentage of Nef-expressing cells that have low (9%) or high (3%) HLA-A2 expression levels. (C) Western blot analysis of Nef and Gag expression from the indicated genome. Lysates were normalized for total protein and percentage of cells positive for intracellular Gag. The immunoblot was probed with an antibody against Nef and then stripped and reprobed with an antibody against Gag. The low level of Gag expression by the molecular clone 94UG114 has been previously reported and is thought to be due to low Rev activity encoded by this genome (3). (D) Determination of the adenoviral MOI (based on 293-cell infectivity) required for efficient CEM T-cell transduction. CEM T cells were transduced with the indicated MOI of adenovirus control (filled curve) or Nef-expressing adenovirus (solid line) and stained for HLA-A2 and CD4. The reduction (*n*-fold) in cell surface expression based on mean fluorescence intensity is indicated. (E) Adenoviral Nef expression levels are comparable to HXB-EP. CEM T cells were transduced with HXB-EP or the indicated MOI of Nef-expressing adenovirus and harvested 72 h later. Lysates normalized for total protein levels and transduction rates were separated by SDS-polyacrylamide gel electrophoresis and then analyzed by Western blotting using an antibody against Nef (AG11, 1:1,000; James Hoxie, University of Pennsylvania) (14, 15).

the transport of newly synthesized MHC-I to the cell surface (16). To determine which domains of Nef were needed for this activity, we performed biochemical transport assays. CEM T cells were first pulse-labeled with ^{35}S-labeled amino acids for 15 min. They were then treated with a biotinylation reagent, which labeled proteins that reached the cell surface during a subsequent 1-h chase period. HLA-A2 was immunoprecipitated and either directly analyzed or reprecipitated with avidin beads to isolate the subset of HLA-A2 molecules that had reached the cell surface.

As expected, we found that wild-type Nef reduced the amount of HLA-A2 transported to the cell surface in 1 h by three- to sixfold (from about 30% to 5%) (Fig. 3A and B). (A reduced yield of total HLA-A2 was obtained in the control

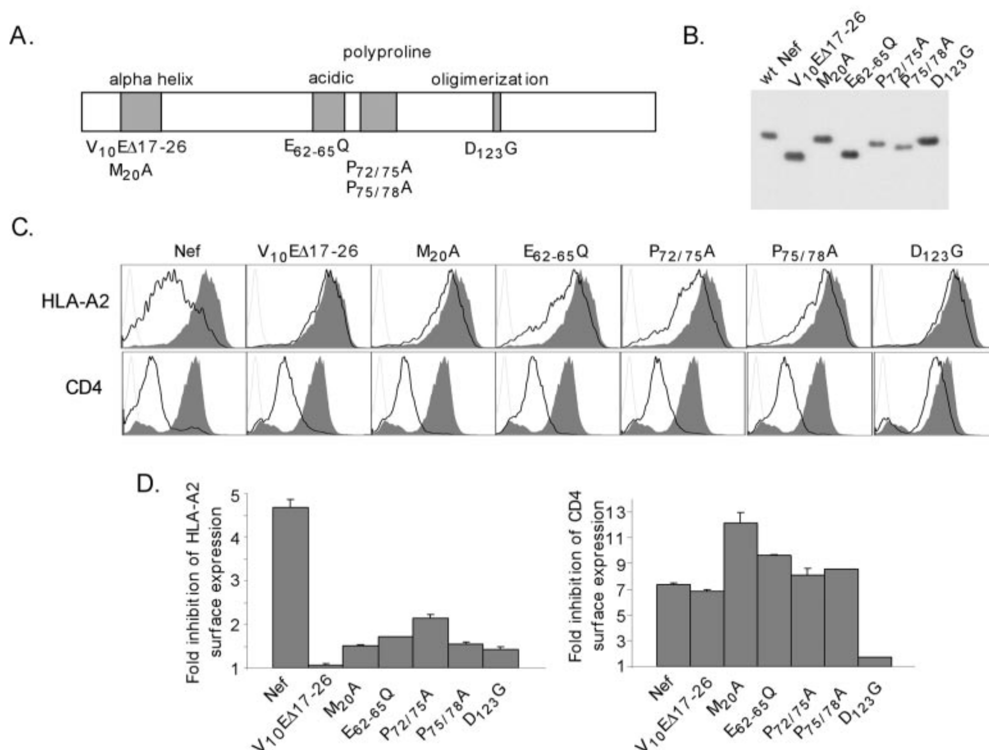

FIG. 2. Analysis of Nef mutants. (A) A schematic diagram of Nef functional domains important for Nef to affect MHC-I trafficking (1, 6, 13, 21, 32). (B) Western blot of NL4-3 Nef expression levels. CEM-A2 T cells were transduced with the indicated adenovirus as previously described (16), with MOIs adjusted so that equal expression was obtained (wild-type Nef, 50:1; $V_{10}E\Delta17$–26, 400:1; $M_{20}A$, 400:1; $E_{62–65}Q$, 600:1; $P_{72/75}A$, 1,000:1; $P_{75/78}A$, 1,000:1; or $D_{123}G$, 400:1). Lysates from transduced cells were immunoblotted and probed with an antibody directed against Nef (2949, 1:5,000; obtained from National Institutes of Health AIDS Repository, contributed by Ronald Swanstrom) (29) and goat anti-rabbit-horseradish peroxidase (Zymed, 1:50,000). (C) The effect of Nef mutants on MHC-I and CD4 cell surface expression. CEM-A2 T cells were transduced as described for panel B, stained 72 h later for either HLA-A2 (BB7.2 [22], 1:100) or CD4 (Serotech, 1:50), and analyzed by flow cytometry. The filled curve represents cells transduced with the control adenovirus, and the solid line represents cells transduced with an adenovirus expressing the indicated Nef construct. (D) The inhibition of cell surface expression (*n*-fold) based on mean fluorescence intensity. The means ± standard deviations of results from two experiments are shown.

sample. However, the fraction that reached the cell surface [33%] was typical of many experiments.) An analysis of Nef mutants revealed that all were defective at disrupting MHC-I transport. Interestingly, the ability of each mutant to block the transport of HLA-A2 correlated with its ability to affect HLA-A2 cell surface expression (compare Fig. 2D with 3B).

Nef has been reported to increase the endocytosis of MHC-I in some cell lines (2, 13, 17, 18, 24, 28). However, under the conditions used in our studies, this activity of Nef is much less apparent in CEM T cells (16) and in HIV-infected primary T cells that express Nef under its normal regulatory elements (Fig. 3C).

In order to affect MHC-I trafficking, Nef binds to specific sequences within the cytoplasmic tail of MHC-I (31). To further define the Nef protein domains required for this step, we tested the ability of wild-type Nef and each Nef mutant to coprecipitate with HLA-A2 in T cells. CEM or CEM-A2 cells were transduced with the indicated adenovirus, as shown in Fig. 2. The cells were incubated in 25 mM NH_4Cl for 4 h and cross-linked with 2 mM dimethyl 3′,3′-dithiobispropionimidate (Pierce). The cells were lysed {phosphate-buffered saline, 0.3% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 0.1% sodium dodecyl sulfate [SDS], pH 8, 1 mM phenylmethylsulfonyl fluoride}, and 1% of the lysates that were normalized for total protein were set aside for input controls. The remaining lysate was precleared with protein A/G agarose and then immunoprecipitated for HLA-A2 using antibody BB7.2 that had been cross-linked to protein A/G agarose (Oncogene) with dimethyl pimelimidate (Sigma). After washing (Tris-buffered saline, 0.3% CHAPS, 0.1% SDS), the cross-linking was reversed, and immunoprecipitates were eluted by incubation in 150 mM dithiothreitol at 37°C for 30 min. The samples were then analyzed by Western blotting using an antibody against Nef.

As shown in Fig. 4A and B, we were readily able to detect wild-type Nef specifically coprecipitating with HLA-A2. However, Nef mutants $V_{10}E\Delta17$-26, $M_{20}A$, and $D_{123}G$ did not coprecipitate with HLA-A2. Assessing the requirement for the polyproline region in MHC-I binding was somewhat more complex, because it appeared more unstable than the other mutants and it was therefore difficult to achieve levels that were equal to that of wild-type Nef. Thus, for these experiments, we utilized two different expression levels of wild-type Nef, one lower than what we typically used in this assay. As shown in Fig. 4B, we found that under conditions in which the expression of wild-type Nef was equal to or less than that of

each mutant, wild-type Nef always coimmunoprecipitated with HLA-A2, while Nef mutants $E_{62\text{-}65}Q$, $P_{72/75}A$, and $P_{75/78}A$ did not. Thus, all of the Nef domains required for reduced MHC-I surface expression were also required for Nef to physically associate with HLA-A2, whereas a domain necessary to affect CD4 but not MHC-I trafficking ($L_{164/165}A$) was not (Fig. 4C).

Some of the Nef domains that we examined have been reported to interact with cellular factors implicated in MHC-I downmodulation. Thus, it was surprising that all of the Nef mutants defective at reducing MHC-I cell surface expression were also defective at interacting with MHC-I. However, it is important to note that our studies do not distinguish whether each Nef domain is needed because of direct interactions with the HLA-A2 cytoplasmic tail or whether it is needed indirectly. For example, the requirement for a particular protein domain in our system could be secondary to nonspecific effects of protein unfolding. Alternatively, it is also possible that some domains are required to interact with cellular factors that stabilize the Nef–MHC-I complex. Finally, it is possible that some domains affect the intracellular localization of Nef. However, in our studies we observed no dramatic change in Nef intracellular localization that would explain the defects we observed (data not shown).

In summary, using a physiologically relevant cell type (T cells), our results demonstrated that Nef mutants that are defective at affecting MHC-I trafficking also failed to inhibit MHC-I export and were unable to coprecipitate with MHC-I. Thus, these studies emphasize the importance of the Nef–MHC-I interaction with respect to Nef's effects on antigen presentation and CTL immune evasion.

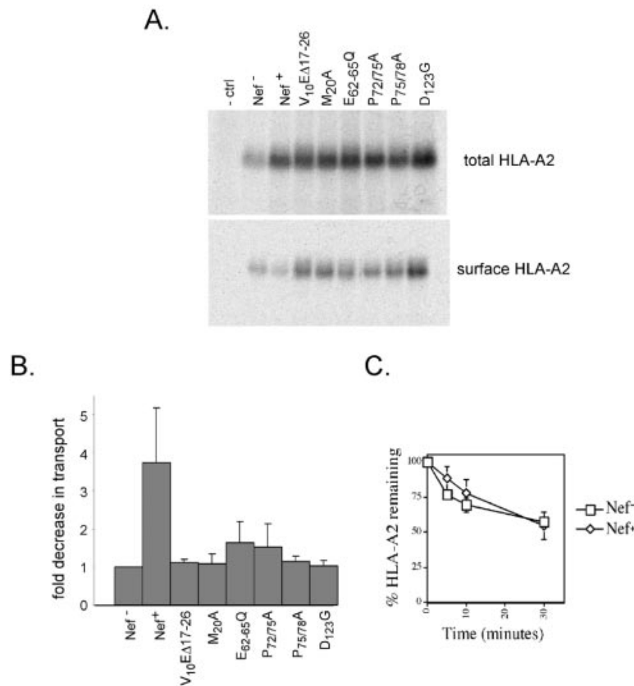

FIG. 3. Nef mutants that are unable to affect HLA-A2 expression do not inhibit the transport of HLA-A2 to the cell surface. (A) CEM T cells transduced with the indicated adenovirus as described in the legend to Fig. 2 were harvested after 3 days, pulse-labeled for 15 min, and chased for 1 h in the presence of a cell-impermeable biotinylation reagent to label cell surface proteins. The cells were lysed and immunoprecipitated with an antibody against HLA-A2. The immunoprecipitates were then eluted by boiling in 10% SDS, and one-third was analyzed directly (total). The remaining two-thirds was reprecipitated with avidin agarose to selectively precipitate the HLA-A2 on the cell surface (surface) before separation by SDS-polyacrylamide gel electrophoresis. Control cells (ctrl) were the parental CEM T cells that did not express HLA-A2. A reduced yield of HLA-A2 was obtained in this Nef^{-} sample, but the fraction that reached the cell surface (33%) was typical of three experiments. (B) The decrease (*n*-fold) in cell surface transport was determined using phosphorimager analysis. The percentage of cell surface HLA-A2 in the Nef^{-} sample was divided by that in the Nef-expressing samples. The means ± standard deviations of results from two separate experiments are shown. (C) Nef does not alter HLA-A2 cell surface stability in HIV-infected primary T cells. The cell surface stability of HLA-A2 in HIV-infected primary T cells was measured by flow cytometry using PLAP as a marker of infection as previously described (16).

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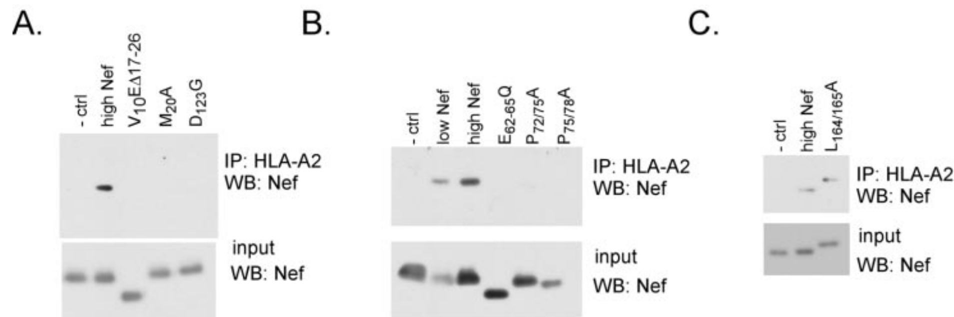

FIG. 4. Nef mutants defective at inhibiting MHC-I cell surface expression do not coprecipitate with HLA-A2. (A, B, and C) CEM T cells were transduced with the indicated adenovirus for 3 days using the MOIs indicated in the legend to Fig. 2 or a 100:1 MOI for HXB $L_{164/165}A$ Nef. The ability of each mutant to coprecipitate with HLA-A2 was assessed by immunoprecipitation and Western blot analysis (top panel) using an antibody directed against Nef (2949, 1:5,000; obtained from National Institutes of Health AIDS Repository, contributed by Ronald Swanstrom) (29) and goat anti-rabbit-horseradish peroxidase (Zymed, 1:50,000). Input controls were incubated in 150 mM dithiothreitol at 37°C for 30 min and analyzed by Western blotting as described above, except a higher dilution of secondary antibody was used (1:200,000). In panel B, two MOIs of wild-type Nef (low [25:1] and high [50:1]) were used because of low expression of some mutants. Control cells (ctrl) were CEM T cells that do not express HLA-A2.

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