

# Nef Association with Human Immunodeficiency Virus Type 1 Virions and Cleavage by the Viral Protease

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**Nef is a regulatory gene product of human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses which enhances virion infectivity by an unknown mechanism. We report here that Nef is detectable at moderate levels in preparations of HIV-1 virions which lack active viral protease (PR). Significantly smaller amounts of intact Nef were present in wild-type virion preparations. Instead, a smaller Nef-related product with an apparent molecular mass of 18 kDa was associated with wild-type virions, indicating that packaging of Nef resulted in cleavage by the viral PR. The presence of the HIV-1 PR inhibitor A77003 during virus production prevented the appearance of the 18-kDa Nef product and caused an accumulation of full-length Nef in virion preparations. Nef associated with comparable efficiency with viral particles produced by the Gag polyproteins of HIV-1 and Moloney murine leukemia virus, indicating that no specific interaction with a virion component is required for the incorporation of Nef. The N-terminal 86 amino acids of Nef were sufficient for packaging into virions. A nonmyristylated form of Nef associated with viral particles with considerably lower efficiency, suggesting that Nef gains access into nascent virions primarily as a consequence of its affinity for membranes. Our results raise the possibility that Nef enhances infectivity directly as a component of the virion.**

The genomes of human immunodeficiency virus type 1 (HIV-1) and other primate lentiviruses contain several open reading frames which are not found in prototypic retroviruses. Among these is the *nef* open reading frame, which is located near the 3' end of the genome and in part overlaps the 3' long terminal repeat. The *nef* gene encodes a 25- to 27-kDa protein expressed early after infection, which is targeted to cellular membranes by the N-terminal attachment of a myristic acid moiety (4, 14). The importance of Nef in vivo was demonstrated by the finding that Nef is required for high virus loads and for the development of AIDS in rhesus macaques infected with SIV<sub>mac</sub> (30). More recently, a requirement for HIV-1 Nef for efficient in vivo viral replication and pathogenicity was demonstrated in the SCID-hu mouse model (27).

Compared to its marked effects on virus spread in vivo, Nef has only relatively moderate effects on virus replication in cell culture. While early reports suggested that Nef suppresses HIV-1 gene expression and virus replication (1, 36, 41, 53), subsequent studies provided evidence for a positive effect of Nef on HIV-1 replication in culture (12, 15, 31, 38, 52, 57). In particular, a significant enhancement of virus replication by Nef was observed when primary CD4<sup>+</sup> cells were exposed to HIV-1 prior to stimulation (38, 52). Recently, several groups have reported that the expression of Nef in virus-producing cells increases the infectivity of viral particles (3, 12, 38, 51). This effect of Nef was observed in different cell types, suggesting that it does not depend on a specific cellular differentiation or activation state (3, 51).

Numerous studies have shown that Nef selectively down-regulates the cell surface expression of CD4 (7, 22, 25, 37). This function of Nef is conserved among different primate

lentiviruses and does not appear to require species-specific factors (7, 21). Nef does not affect the biosynthesis or the transport of CD4 through the secretory pathway to the plasma membrane but, instead, induces CD4 endocytosis and subsequent degradation in an acidic compartment (2, 45, 47, 50). The study of chimeric molecules demonstrated that the cytoplasmic domain of CD4 is both required and sufficient for Nef-induced down-regulation (2, 5, 6, 21). However, specific residues in the CD4 cytoplasmic domain which are required for p56<sup>lck</sup> binding or for phorbol ester-induced CD4 endocytosis are dispensable for Nef-induced down-regulation (2, 22). The effect of Nef on CD4 surface levels in acutely infected T cells is manifest at an early stage, before Env-mediated receptor down-regulation can be observed (2).

While it is conceivable that Nef augments virion infectivity by reducing the potential for inappropriate interactions between Env and CD4 during virus egress, several lines of evidence argue against this possibility. First, the Nef-mediated enhancement of virion infectivity is independent of the Env-CD4 interaction, because it is observed even after pseudotyping of Env-deficient HIV-1 virions with amphotropic murine leukemia virus Env proteins (3, 39). Second, the infectivity of Env-deficient virus could be enhanced by providing Nef in producer cells regardless of whether CD4 was expressed or not (3, 38). Third, Nef enhanced the growth rate of HIV-1 in cells expressing a C-terminally truncated CD4 which was not internalized by Nef (11). Fourth, a conserved proline repeat in Nef which is critical for the enhancement of HIV-1 virion infectivity is dispensable for CD4 down-regulation (23, 46).

To stimulate virion infectivity, Nef must associate with the cell membrane, where virus assembly occurs (12, 23). We therefore investigated the possibility that Nef gains access into virions. We report here that Nef does associate with viral particles and is processed to an 18-kDa form during virion maturation.

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## MATERIALS AND METHODS

**Plasmids.** The ptrENV vector is a variant of the HIV-1<sub>BRU/LAI</sub> infectious clone pBRU-1 with large deletions in *gag*, *pol*, and *env* (44). Because ptrENV harbors a frameshift mutation after codon 86 of the *nef* gene, a *nef*-positive version, ptNef<sup>+</sup>, was generated by replacing a segment between a unique *Bam*HI site and a *Bsp*EI site (nucleotides [nt] 8478 to 9396 of HIV-1) with the corresponding sequence from the infectious molecular clone pLAI (43). A *nef*-negative version, ptNef<sup>-</sup>, was obtained by inserting 4 bp at the *Xho*I site in the *nef* gene of ptrENV by using the Klenow fragment of DNA polymerase I. This manipulation generated an additional frameshift mutation after codon 35 of the *nef* gene. The ptNef<sup>mut2</sup> vector is a version of ptNef<sup>+</sup> in which the codon for glycine 2 of Nef has been replaced by site-directed mutagenesis by a codon specifying alanine. It was previously shown that this substitution prevents the myristylation of Nef (55).

The full-length HIV-1 proviral constructs HXB-PR<sup>-</sup> (24) and HXB-*gag*<sup>-</sup> (16) are identical to the *nef*-negative HXBH10 infectious clone of HIV-1 (24), except for mutations that inactivate protease (PR) or prevent the expression of the Gag polyprotein Pr55<sup>gag</sup>. LAI-PR<sup>-</sup> and DFCI-HT-PR<sup>-</sup> are PR-defective versions of the *nef*-positive proviral clones pLAI (43) and DFCI-HT (33), respectively. These constructs were obtained by replacing a segment between unique *Apa*I and *Sal*I sites (nt 2010 to 5789) with the corresponding segment from HXB/Vpr<sup>+</sup>/PR<sup>-</sup> (9), a *vpr*-positive version of HXB-PR<sup>-</sup>.

The HXBH10-based *nef*-positive proviral construct HXB/Nef<sup>+</sup> was obtained by replacing a segment between a unique *Sal*I site and a *Bsp*EI site (nt 5789 to 9396) in HXBH10 with the corresponding segment from pLAI. HXB/Nef<sup>+</sup>/*gag*<sup>-</sup> is a variant of HXB/Nef<sup>+</sup> which is unable to express Pr55<sup>gag</sup> because of a premature termination codon in place of codon 8 of the *gag* gene and an additional frameshift mutation in the CA coding region (16). HXB/Nef<sup>+</sup>/PR<sup>-</sup> is a PR-defective variant of HXB/Nef<sup>+</sup> which has the codon for Asp-25 of HIV-1 PR replaced by a codon specifying glutamic acid. HXB/Nef<sup>-</sup> and HXB/Nef<sup>-</sup>/PR<sup>-</sup> are *nef*-negative versions of HXB/Nef<sup>+</sup> and HXB/Nef<sup>+</sup>/PR<sup>-</sup>, respectively, which were obtained by inserting 4 bp at the unique *Xho*I site in the *nef* gene (nt 8900) by using the Klenow fragment of DNA polymerase I.

HXB/NCA<sub>gag</sub><sup>-</sup>/PR<sup>-</sup> (32) contains the Moloney murine leukemia virus (MLV) *gag* gene in a *nef*-negative HIV-1 background and was used to express the MLV Gag polyprotein Pr65<sup>gag</sup>.

**Cell culture and transfections.** COS-7, HeLa, and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (10<sup>6</sup>) were seeded into 80-cm<sup>2</sup> tissue culture flasks 24 h prior to transfection. Cultures were transfected with proviral plasmid DNA by a calcium phosphate precipitation technique (13).

**Viral protein analysis.** Transfected cells were metabolically labeled with [<sup>35</sup>S]methionine (50 μCi/ml) from 48 to 60 h posttransfection. Labeled cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), and Nef proteins were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The sera used for immunoprecipitation were a rabbit polyclonal Nef-specific antiserum raised against the LAI Nef protein made in *Escherichia coli* (56) and a rabbit anti-Nef serum raised against a recombinant BH10 Nef protein (8, 40).

Viral particles released during the labeling period were pelleted through sucrose cushions (in phosphate-buffered saline) for 90 min at 4°C and 26,000 rpm in a Beckman SW28 rotor. Pelleted virions were lysed in RIPA buffer, and viral proteins were either directly analyzed by SDS-PAGE or immunoprecipitated prior to electrophoresis with the rabbit anti-Nef serum raised against LAI Nef protein (56).

## RESULTS

**Association of truncated Nef with HIV-1 virions.** We have previously shown that ptrENV, an HIV-1<sub>BRU/LAI</sub> proviral clone with large deletions in the *gag*, *pol*, and *env* genes (44), can supply Vpr in *trans* for packaging into HIV-1 virions (32). During the course of those studies, we noted that ptrENV transfection resulted in the appearance of a second virion-associated protein which migrated slightly faster than did Vpr during SDS-PAGE. In these experiments, virions were produced in COS-7 cells transfected with the full-length, PR-defective HIV-1 proviral clone HXB-PR<sup>-</sup>, which is *vpr* positive but lacks open reading frames for the auxiliary genes *vpr* and *nef*. After metabolic labeling with [<sup>35</sup>S]methionine from 48 to 60 h posttransfection, virions released from transfected cells were partially purified by ultracentrifugation through 20% sucrose. Pelleted material was lysed in RIPA buffer and analyzed directly by SDS-PAGE and autoradiography. Cotransfection of ptrENV resulted in the appearance of Vpr and of a novel protein with an apparent molecular mass of about 10 kDa in

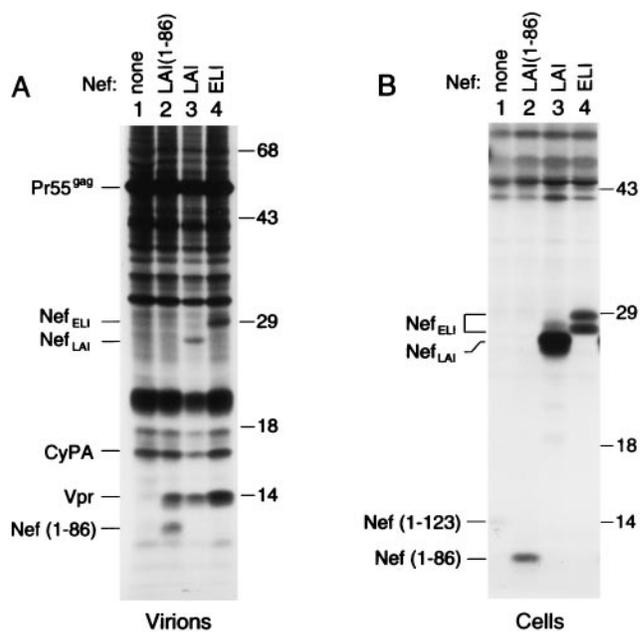


FIG. 1. Association of truncated and full-length Nef with HIV-1 virions. (A) Sedimentation of particulate material from culture supernatants. Virions released into the supernatant were pelleted through 20% sucrose, disrupted in RIPA buffer, and analyzed directly by SDS-PAGE. (B) Immunoprecipitation from cell lysates with a rabbit anti-Nef serum raised against LAI Nef. COS-7 cells were transfected with the PR-defective, *nef*-negative HIV-1 proviral construct HXB-PR<sup>-</sup> (lane 1), HXB-PR<sup>-</sup> plus ptrENV (lane 2), or the PR-defective, *nef*-positive HIV-1 proviral constructs LAI-PR<sup>-</sup> (lane 3) and DFCI-HT-PR<sup>-</sup> (lane 4). Transfected cells were metabolically labeled with [<sup>35</sup>S]methionine for 12 h. The positions of migration of molecular mass markers (in kilodaltons) are indicated on the right. The positions of specific full-length and truncated proteins are indicated on the left. CyPA, cyclophilin A.

viral particle preparations (Fig. 1A, lane 2). Neither Vpr nor the 10-kDa protein was seen after transfection of HXB-PR<sup>-</sup> alone (Fig. 1A, lane 1). Further analysis showed that a frameshift mutation in the *vpr* gene of ptrENV which prevented the virion incorporation of Vpr had no effect on the association of the 10-kDa protein with viral particles; in contrast, a frameshift mutation at an *Xho*I site in the ptrENV *nef* gene prevented the appearance of the 10-kDa protein (data not shown).

DNA sequence analysis of ptrENV revealed the presence of a frameshift mutation at a *Bgl*II site in the *nef* gene due to the insertion of 4 bp. As a result, ptrENV encodes only the first 86 of the 206 amino acids of the LAI Nef protein, followed by 2 unrelated residues. To determine whether the truncated Nef protein was stably expressed, Nef-related proteins were immunoprecipitated from cell lysates with different antisera raised against recombinant Nef. Small amounts of a protein of about 14 kDa, representing the C-terminally truncated 123-amino-acid HXB2 Nef product, were precipitated from cells transfected with HXB-PR<sup>-</sup> alone (Fig. 1B, lane 1, and data not shown). Cotransfection of ptrENV yielded a more prominent Nef-reactive species of about 10 kDa (Fig. 1B, lane 2), the expected molecular mass of the truncated LAI Nef protein encoded by ptrENV. The 10-kDa Nef species expressed by ptrENV, as well as a trace amount of the 14-kDa species encoded by HXB-PR<sup>-</sup>, could also be immunoprecipitated from sucrose-purified virion preparations (data not shown).

**Full-length Nef associates with HIV-1 virions.** The appearance of N-terminal Nef fragments in viral particle preparations raised the possibility that full-length Nef gains access into virions. To examine this possibility, we used PR-defective variants

of the *nef*-positive pLAI and DFCI-HT proviruses, which harbor the widely divergent LAI and ELI *nef* alleles, respectively. The proviral constructs were transfected into COS-7 cells, and the expression of Nef was confirmed by immunoprecipitation with a rabbit polyclonal antiserum raised against the LAI Nef protein. A 25-kDa Nef protein was specifically immunoprecipitated from lysates of cells transfected with the LAI-PR<sup>-</sup> construct (Fig. 1B, lane 3). The DFCI-HT-PR<sup>-</sup> proviral construct, which harbors the ELI *nef* allele, yielded two Nef protein species of approximately 27 and 29 kDa (Fig. 1B, lane 4). Similar differences in apparent molecular mass between the LAI and ELI Nef proteins have previously been reported (58). It was shown that Nef proteins that have an alanine at position 54, such as the LAI Nef protein, migrate faster during SDS-PAGE than do proteins, such as ELI Nef, which have an aspartic acid at position 54 (42).

To compare the protein contents of virions produced by LAI-PR<sup>-</sup> and DFCI-HT-PR<sup>-</sup>, [<sup>35</sup>S]methionine-labeled particulate material released into the supernatant of transfected COS-7 cells was pelleted through 20% sucrose, solubilized in RIPA buffer, and analyzed directly by SDS-PAGE and autoradiography. Virions produced by the LAI-PR<sup>-</sup> construct contained small amounts of a 25-kDa protein (Fig. 1A, lane 3) which comigrated with LAI Nef immunoprecipitated from cell lysates. The 25-kDa protein was not present in virions produced by the DFCI-HT-PR<sup>-</sup> construct (Fig. 1A, lane 4). Instead, a protein which comigrated with the 29-kDa ELI Nef protein species was detected (Fig. 1A, lane 4). Immunoprecipitation with anti-Nef serum confirmed that the 25- and 29-kDa proteins detected in viral particle preparations represented LAI Nef and ELI Nef, respectively (see Fig. 2 and 3) (data not shown). The amounts of particle-associated Nef were compared by scanning densitometry to those of another auxiliary viral protein, Vpr, which is known to be incorporated into virions (32, 35). These comparisons indicated that the molar amounts of Nef in viral particle preparations were at least 10- to 15-fold lower than those of Vpr.

As shown in Fig. 2A, the amount of Nef in the particulate fraction was reduced by more than 90% when the *gag* gene of a *nef*-positive HIV-1 proviral construct was disrupted. This result indicates that only a minor fraction of the Nef present in viral particle preparations was due to contamination by cellular debris (see also Fig. 3A, lane 2). A nonmyristylated form of Nef associated with viral particles with about 10-fold-lower efficiency than did wild-type Nef, as determined by comparing the intensities of mutant and wild-type Nef protein bands in cell lysates and particulate fractions by scanning densitometry (Fig. 2B).

**Nef associates with heterologous viral particles.** We and others have shown that the virion incorporation of the auxiliary viral protein Vpr is mediated by the C-terminal p6 domain of the HIV-1 Gag polyprotein (32, 35). To determine whether the virion association of Nef also requires a homologous virion component, we tested whether Nef can associate with viral particles formed by the MLV Gag polyprotein Pr65<sup>gag</sup>. LAI Nef was supplied in *trans* by ptNef<sup>+</sup>. Cotransfection of ptNef<sup>+</sup> and of the PR-defective, *nef*-negative HIV-1 proviral construct HXB/Vpr<sup>+</sup>/PR<sup>-</sup> into HeLa cells yielded immature HIV-1 virions which contained a 25-kDa protein that was not present in virions produced after transfection of HXB/Vpr<sup>+</sup>/PR<sup>-</sup> alone (Fig. 3A, lanes 3 and 4). The 25-kDa protein was also present in immature MLV particles produced after cotransfection of ptNef<sup>+</sup> and HXB/NCA<sub>gag</sub>-PR<sup>-</sup>, an MLV Gag polyprotein expression construct that contains the MLV *gag* gene in a *nef*-negative HIV-1 background (Fig. 3A, lane 5). The 25-kDa protein could be immunoprecipitated from both HIV-1 and

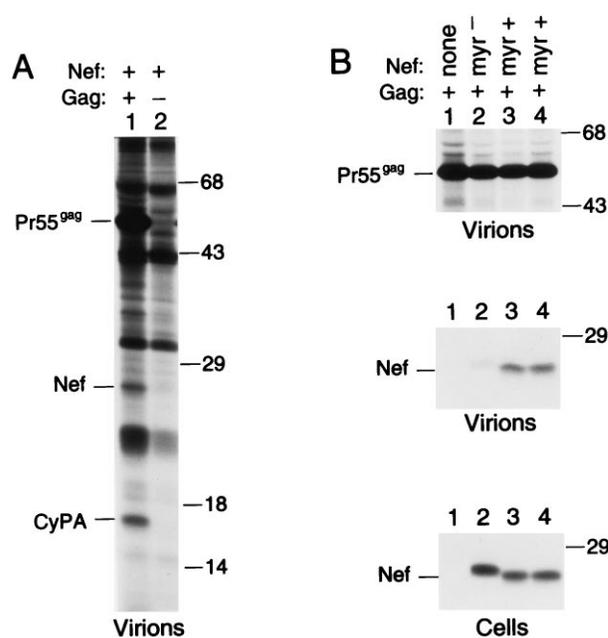


FIG. 2. Requirement for Gag expression for the appearance of Nef in the particulate fraction and role of Nef myristylation. (A) COS-7 cells were transfected with the PR-defective, *nef*-positive proviral construct HXB/Nef<sup>+</sup>/PR<sup>-</sup> (lane 1) and HXB/Nef<sup>+</sup>/gag<sup>-</sup>, a variant which is unable to form viral particles (lane 2). (B) HeLa cells were cotransfected with the PR-defective, *nef*-negative proviral construct HXB/Vpr<sup>+</sup>/PR<sup>-</sup> and either ptNef<sup>-</sup> (lane 1), ptNef<sup>mut2</sup> (lane 2), or ptNef<sup>+</sup> (lanes 3 and 4). In panel A, transfected cells were metabolically labeled with [<sup>35</sup>S]methionine, followed by sedimentation of released particulate material through 20% sucrose and direct analysis by SDS-PAGE and autoradiography. In panel B, aliquots of the pelleted material were either analyzed directly by SDS-PAGE to compare the amounts of particulate Gag protein in the samples (top) or immunoprecipitated with anti-Nef serum prior to SDS-PAGE (middle). Nef was also immunoprecipitated from the lysates of transfected cells to compare intracellular steady-state levels (bottom). The positions of migration of molecular mass markers (in kilodaltons) are indicated on the right. The positions of specific proteins are indicated on the left. myr<sup>+</sup>, myristylated form; myr<sup>-</sup>, nonmyristylated form. CyPA, cyclophilin A.

MLV particles with antiserum directed against LAI Nef protein, confirming its identity (Fig. 3A [bottom]). MLV particles contained about 1.3-fold-more Nef than did HIV-1 particles, as calculated from a comparison of the intensities of the Nef and Gag protein bands in Fig. 3A by scanning densitometry, taking into account the number of methionines in Pr55<sup>gag</sup> and Pr65<sup>gag</sup> (15 versus 3). Thus, Nef can associate with comparable efficiency with viral particles formed by the Gag polyproteins of widely divergent retroviruses.

**Virion-associated Nef is cleaved by PR.** To examine whether Nef can be detected in mature HIV-1 virions, we constructed a *nef*-positive variant of the infectious molecular clone HXBH10, HXB/Nef<sup>+</sup>. To produce an otherwise isogenic provirus that lacked a *nef* open reading frame, a frameshift mutation was introduced at a unique *Xho*I site in the *nef* gene of HXB/Nef<sup>+</sup> to yield HXB/Nef<sup>-</sup>. Variants of HXB/Nef<sup>+</sup> and HXB/Nef<sup>-</sup> which are incapable of making active PR were also constructed and called HXB/Nef<sup>+</sup>/PR<sup>-</sup> and HXB/Nef<sup>-</sup>/PR<sup>-</sup>, respectively. As expected, transfection of the HXB/Nef<sup>+</sup>/PR<sup>-</sup> construct into COS-7 cells yielded immature viral particles which contained the 25-kDa LAI Nef protein (Fig. 3B, lane 5). In contrast, mature virions produced by the HXB/Nef<sup>+</sup> construct contained hardly any 25-kDa Nef protein, as determined by immunoprecipitation from virion lysates. Instead, a faster-migrating Nef-reactive species of about 18 kDa which was not

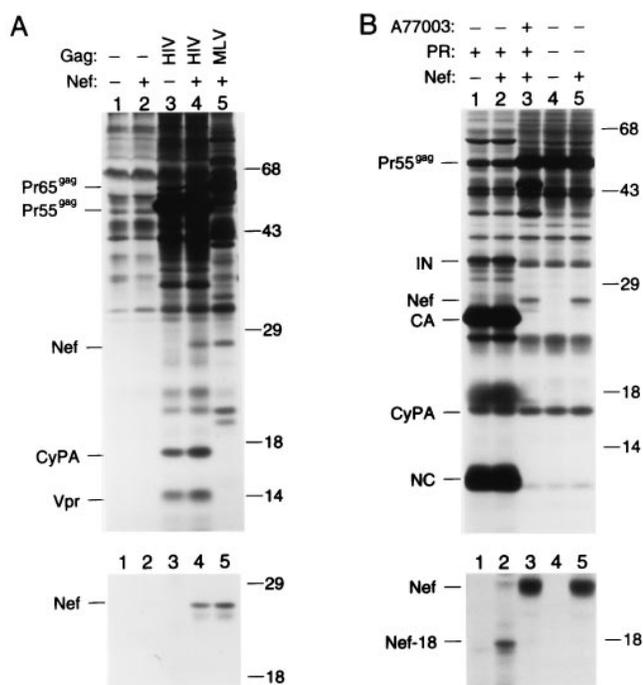


FIG. 3. Association of Nef with heterologous viral particles and cleavage by PR. (A) HeLa cells were cotransfected with  $\text{ptNef}^-$  and the  $\text{nef}$ -negative HIV-1 proviral construct HXB- $\text{gag}^-$  (which is unable to form viral particles) (lane 1),  $\text{ptNef}^+$  and HXB- $\text{gag}^-$  (lane 2),  $\text{ptNef}^-$  and the PR-defective,  $\text{nef}$ -negative HIV-1 proviral construct HXB/Vpr $^+$ /PR $^-$  (lane 3),  $\text{ptNef}^+$  and HXB/Vpr $^+$ /PR $^-$  (lane 4), or  $\text{ptNef}^+$  and the MLV Gag polyprotein expression construct HXB/NCA $_{\text{gag}}^-$ /PR $^-$  (lane 5). (B) COS-7 cells were transfected with the HIV-1 proviral constructs HXB/Nef $^-$  (lane 1) and HXB/Nef $^+$  (lanes 2 and 3) or the PR-defective versions HXB/Nef $^-$ /PR $^-$  (lane 4) and HXB/Nef $^+$ /PR $^-$  (lane 5). Cells transfected with HXB/Nef $^+$  were maintained in the absence (-; lane 2) or presence (+; lane 3) of 10  $\mu\text{M}$  A77003 during metabolic labeling. Transfected cells were metabolically labeled with [ $^{35}\text{S}$ ]methionine for 12 h, and viral particles released during the labeling interval were analyzed directly by SDS-PAGE and autoradiography (top) and in parallel by immunoprecipitation with anti-Nef serum prior to SDS-PAGE (bottom). The positions of migration of molecular mass markers (in kilodaltons) are indicated on the right. The positions of specific proteins are indicated on the left. Nef-18, 18-kDa Nef species. IN, integrase; CA, capsid protein; NC, nucleocapsid protein; CyPA, cyclophilin A.

present in virions produced by the HXB/Nef $^-$  construct was detected (Fig. 3B, bottom, lanes 1 and 2). Because of a diffuse band of unknown identity which comigrated with the 18-kDa Nef, this Nef species could not be discerned when mature virion lysates were directly analyzed by SDS-PAGE (Fig. 2B [top]).

Since HXB/Nef $^+$  and HXB/Nef $^+$ /PR $^-$  differ only in the PR coding region, these results indicate that Nef is processed by PR upon incorporation into virions. To confirm this finding, we tested the effects of A77003, a specific inhibitor of HIV-1 PR (29), on the appearance of the 18-kDa Nef species in viral particles. To this end, COS-7 cells transfected with HXB/Nef $^+$  were metabolically labeled for 12 h in the presence or absence of A77003. At a concentration of 10  $\mu\text{M}$ , the drug largely prevented Gag polyprotein processing, as expected (Fig. 3B, lane 3). Interestingly, the drug also prevented the appearance of the 18-kDa Nef species in particles produced by the HXB/Nef $^+$  construct and caused an accumulation of the 25-kDa species to levels seen in PR-defective virions (Fig. 3B, lanes 3 and 5). Similar results were obtained when viral particles were produced in HeLa and 293T cells (data not shown).

In addition to the 25-kDa LAI Nef protein, a trace amount

of the 18-kDa Nef species was also detectable in the lysate of COS-7 cells transfected with HXB/Nef $^+$ . However, the 18-kDa species was not seen after mutational inactivation of PR or after maintenance of cells transfected with the parental construct in the presence of the PR inhibitor A77003 (data not shown).

## DISCUSSION

Nef has been shown to augment HIV-1 replication by enhancing virion infectivity (3, 11, 12, 23, 38, 39, 51). HIV-1 virions produced in the absence of Nef enter target cells efficiently but show a reduced ability to reverse transcribe the viral RNA genome once internalized (3, 11, 51). Furthermore, it has been reported that Nef stimulates virion infectivity when provided in *trans* in producer cells but not in target cells (3, 39). Since Nef has no apparent effect during virus morphogenesis, these findings suggest that it may act as a component of the virion. However, an early study failed to detect Nef in sucrose-purified HIV-1 virion preparations (18).

Here we provide evidence that Nef is incorporated into HIV-1 virions in small quantities and that virion-associated Nef is cleaved by PR. We find that the predominant Nef species in mature virions is a processed form of 18 kDa, possibly explaining why previous attempts to determine whether Nef is present in virions yielded equivocal results (39). In contrast to mature virions, immature HIV-1 virions produced in the presence of a specific PR inhibitor or after mutational inactivation of PR contained only full-length Nef. These results imply that the conversion of full-length Nef to the virion-associated 18-kDa form is mediated by PR. Furthermore, the finding that preparations of mature virions contained mostly the processed form indicates that Nef is indeed incorporated into viral particles, which provide an environment with a high local concentration of active PR. In contrast, in cell lysates, the processed 18-kDa Nef species represented only a very minor form, perhaps reflecting the presence of budded but not yet fully released viral particles at the cell surface.

Although this study did not specifically address the mechanism by which Nef gains access into virions, some conclusions can be drawn. We have shown that Nef expressed in *trans* can associate with comparable efficiency with HIV-1 virions and with viral particles produced by the Gag polyprotein of MLV, which does not encode a Nef-like protein. Particles produced by the MLV Gag polyprotein expression construct lacked other virion components, such as reverse transcriptase and integrase, demonstrating that these components are dispensable for the incorporation of Nef. Furthermore, because the Gag proteins of HIV-1 and MLV exhibit very little sequence homology, our observations argue against a requirement for a specific interaction with Gag. The requirements for the virion association of Nef clearly differ from those for the incorporation of Vpr or the host cell protein cyclophilin A, which are both recruited by specific HIV-1 Gag domains (19, 32, 35, 54).

N-terminal myristylation targets Nef to the cell membrane (28, 55), the site where HIV-1 virion assembly occurs (26). The myristylation of Nef is crucial both for its ability to down-regulate CD4 and for its effect on virion infectivity (2, 3, 12, 23). We have found that myristylation is also crucial for the association of Nef with virions. It is noteworthy in this respect that a truncated Nef which retained only the N-terminal 86 amino acids, including the myristic acid attachment site, was incorporated into virions at least as efficiently as was full-length Nef. These observations suggest that association with the cell membrane, primarily as a consequence of N-terminal myristy-

lation (28, 55), may be sufficient for the inclusion of Nef into nascent viral particles.

While Nef is packaged nonspecifically and at relatively low levels compared to those of Vpr, its presence in HIV-1 virions may nevertheless be biologically relevant. Several reports have indicated that Nef associates with cellular kinases (17, 34, 46, 48, 49), raising the possibility that these enzymes play a role in the Nef-mediated enhancement of virion infectivity. HIV-1 Nef contains a conserved proline-rich sequence which binds to the SH3 domains of a subset of Src family tyrosine kinases, including the SH3 domain of Hck (34, 46). Indeed, mutations in this proline-rich region which disrupted the Nef-Hck interaction also abrogated the ability of Nef to stimulate virus replication but had no effect on Nef-induced CD4 down-regulation (46). In addition to tyrosine kinases, Nef binds a cellular serine kinase, and both the N-terminal membrane targeting signal of Nef and a central highly conserved region are critical for this interaction (48, 49).

Interestingly, it was recently reported that tyrosine, serine, and threonine kinase activities are present in HIV-1 virions and that the viral matrix protein is a substrate for these kinases (10, 20). Phosphorylation of the matrix protein has been reported to be critical for virion infectivity, particularly for the infection of nondividing cells such as terminally differentiated macrophages (10, 20). It is conceivable that Nef remains bound to cellular kinases during its incorporation into viral particles and may subsequently be cleaved by PR to release associated cellular proteins from the lipid envelope of the virion. In this model, Nef would affect virion infectivity indirectly by recruiting cellular enzymatic activities, which in turn modify virion components such as the matrix protein. In this regard, it will be of interest to determine whether the virion association of cellular kinases depends on the expression of Nef.

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