Interactions of Processed Nef (58-206) with Virion Proteins of HIV Type 1

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ABSTRACT

The Nef protein plays a major role *in vivo* in promoting HIV and SIV replication and pathogenesis. *In vitro*, Nef has been shown to down-regulate cell surface molecules, such as CD4 and MHC-I, alter T cell signaling, and enhance virion infectivity. These effects are attributed to interactions of Nef with cellular proteins. In addition, HIV Nef is incorporated into viral particles, mainly localizing in the virion cores. However, no report has been published to date regarding Nef interactions with virion proteins. By immunoprecipitation, Nef was found to bind to viral enzymes. Using yeast two-hybrid and GST pulldown procedures to find out direct potential partners of Nef, Nef was consistently found to interact with viral integrase (IN). The interaction between Nef and IN was stronger when Nef was present as the viral protease-cleaved isoform. We hypothesize that the interaction of Nef with viral integrase or other virion proteins may explain the presence of Nef in viral cores. In addition, this interaction suggests that Nef may accompany the reverse transcription and the preintegration complexes during the early steps of the infection cycle and potentially affect infectivity during these steps.

INTRODUCTION

NEF, A PROTEIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV), plays an important role in primate lentivirus virulence. The absence of a functional *nef* gene in strains infecting human or rhesus monkeys results in low viral loads and significantly slows clinical progression of disease.[1,2] Furthermore, expression of *nef* as a transgene in mice reproduces many of the pathological effects seen in AIDS.[3] *In vitro* studies demonstrated that Nef can affect multiple cellular functions that help explain its modulating effect on pathogenicity.[4–12] Nef mediates down-regulation of the cell surface expression of CD4 molecules[13–15] and major histocompatibility complex (MHC) class I expression[16,17] shielding infected cells from cytotoxic T lymphocyte attack.[18–20] Nef alters cell signaling pathways,[21,22] and induces chemokine release from infected macrophages.[23]

A key feature of Nef influence on pathogenesis is that Nef increases HIV-1 replication by enhancing the infectivity of virions by approximately 5- to 10-fold in single-cycle infection assays.[24–28] The effects of Nef on virion infectivity involve both CD4-dependent and CD4-independent components.[29–31] By down-regulating the CD4 molecules at the cell surface, Nef prevents CD4 interference with Env incorporation on particles, enhancing the infectivity of viruses budding from CD4^{+} cells.[32] On the other hand, Nef^{+} virions still display about 2-fold enhanced infectivity as compared with Nef^{−} virions when produced from CD4^{−} cells, suggesting that Nef affects virion infectivity in a CD4-independent manner as well[32] (our unpublished results). The fact that Nef-defective viruses can achieve nearly wild-type levels of infectivity when produced in cells where Nef is provided in *trans* suggests that Nef modifies the virion either directly or indirectly.[33,34] Indeed, 10 to 100 Nef molecules have been detected in viral particles,[35] mainly localizing to the viral cores.[36] In the viral particle, Nef is found as a full-length 27-kDa protein and as a 20-kDa protein, resulting from cleavage between residues W[57] and L[58] by the viral protease.[35,37–39] However, Nef cleavage does not seem to be required for the enhancement of infectivity,[40–42] leaving the functional importance of Nef processing to be determined.

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Taken together, these reports suggest that the CD4-independent effect of Nef on virion infectivity is mediated by Nef incorporation into viral particles. Nef might act through the recruitment of a kinase at the viral budding site in the producer cell, that in turn could modify the virion. Alternatively, Nef might act per se in the viral core and affect directly postentry steps, possibly at the level of viral uncoating or reverse transcription.

Nef is a membrane-anchored protein. Its presence in viral cores suggests that it might be recruited there by binding to core proteins. In preliminary experiments, we observed that the immunoprecipitation of labeled virion lysates using anti-Nef antibodies brought down a range of proteins with molecular weights consistent with viral core proteins. This suggested that Nef was bound to core proteins in these lysates.

We have therefore investigated Nef interactions with viral core proteins, using coimmunoprecipitation, yeast two-hybrid, and *in vitro* binding assays. We propose that Nef interacts directly with core proteins, particularly with the viral integrase, and that this interaction may explain the presence of Nef in the virion core.

MATERIALS AND METHODS

Cells and viruses

CEM-GFP cells (CEM T cell line stably transfected with GFP under the control of the HIV LTR; NIH AIDS Research and Reference Reagent Program, USA; kindly provided by Dr. Alain Gervaix, University Hospital, Geneva, Switzerland)[43] were cultured in RPMI 1640 medium (Gibco, Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS) and 50 μg/ml gentamycin.

293T cells were cultured in Dulbecco's modified Eagle minimal essential medium (DMEM; Gibco, Invitrogen), supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamycin. To express Nef in the context of the whole virus genome, the proviral DNA constructs, pNL4-3 (GenBank accession number M19921)[44] and pNL4-3Δ*nef* (kindly provided by Dr. Douglas Richman, University of California, San Diego)[27] were used to transfect cells and to produce viruses. Deletion of 1805 bp in the *pol* gene (RT and IN orf) was performed by cutting out the *Xmn*I fragment (pos. 2683 and 4488) in pNL4-3, generating the plasmid pNL4-3ΔRTΔIN. The construction pNL4-3ΔPR contains a deletion in the PR orf.[45] CEM-GFP cells (10^6 cells) were transfected with 3 μg of plasmid DNA and 9 μl of Transfast reagent (Promega, Catalys) in a six-well plate in 1 ml RPMI 1640 medium for 1.5 hr. Complete medium was then added and cells were cultured for 2 weeks with regular cell passages. 293T cells were transfected by the calcium phosphate technique with 20 μg of plasmid DNA.

Virus-containing supernatants were harvested and cleaned from cell debris by centrifugation and 0.22-μm filtration. Virion concentration was assessed by measuring the p24 (CA) antigen by ELISA (HIVAG-1; Abbott Laboratories), according to the manufacturer's protocol.

Antibodies

All HIV-specific antibodies were provided by the NIH AIDS Research and Reference Reagent Program (Rockville, MD): human anti-HIV-1 immunoglobulins (Ref. 3957), rabbit HIV-1 Nef antiserum (Ref. 2949), rabbit HIV-1 matrix (MA) antiserum (Ref. 4811), sheep HIV-1 capsid (CA) antiserum (Ref. 287), rabbit HIV-1 integrase (IN) antiserum (Ref. 756), mouse HIV-1 reverse transcriptase (RT) antiserum (Ref. 3483), rabbit HIV-1 protease (PR) antiserum (Ref. 4105), rabbit HIV-1 Vpr antiserum (Ref. 3951), rabbit HIV-1 Vif antiserum (Ref. 2221), rabbit HIV-1 Vpu antiserum (Ref. 969), and human HIV-1 gp41 antiserum (Ref. 1475). Sheep HIV-1 Nef antiserum, used for Nef immunoblottings, was kindly provided by Dr. John Guatelli (University of California, San Diego).[35] Mouse anti-LexA antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-HA (influenza hemagglutinin) antiserum (Roche), as well as horseradish peroxidase (HRP)-conjugated antisheep, antimouse, or antirabbit immunoglobulins (Dako) were obtained commercially.

Immunoprecipitation

As antisheep IgG antibodies used as secondary antibodies in Western blots reacted with the antibodies used for immunoprecipitation, then leading to the detection of prominent bands corresponding to the light and heavy Ig chains, we attempted to prevent this phenomenon by covalently coupling the immunoprecipitating antibodies to the protein A beads as described.[46] Briefly, 2 mg of antibody and 1 mg of protein A-Sepharose were incubated at room temperature for 1 hr. The Ig-protein A beads were then washed twice with 10 vol 0.1 M sodium borate (pH 9.0) and resuspended in the same buffer supplemented with 20 mM dimethylpimelimidate (solid) for another 30 min to allow covalent binding between antibody and protein A beads. The reaction was stopped by washing with 0.2 M ethanolamine (pH 8.0) and incubating in the same solution for 2 hr. After an additional wash with 0.2 M ethanolamine, the beads were resuspended in phosphate-buffered saline (PBS)–0.1% thimerosal and stored at 4°C until use.[46]

Virion-containing supernatants were produced as described above and then ultracentrifuged for 90 min with $100,000 \times g$ at 4°C through a 5-ml PBS–5% sucrose cushion. Pelleted viruses were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) for 30 min at 4°C in the presence of protease inhibitors (Roche, Cat. 1697498, "Complete" protease inhibitors cocktail). Samples were precleared by incubation with 50 μl protein A-Sepharose (Amersham Pharmacia) and 5 μl normal human, rabbit, or mouse serum for 2 hr at 4°C. Samples were then centrifuged and the supernatants were incubated with 50 μl protein A-Sepharose directly coupled to various antisera (anti-HIV, anti-Nef, anti-MA, anti-IN, anti-RT, anti-PR, anti-Vpr) overnight at 4°C. Protein A beads were washed three times with RIPA buffer and protein samples were eluted in 2$\times$ Gel Sample Buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.05% bromophenol blue) for 10 min at 95°C.

Immunoblot analysis

Immunoprecipitated samples were separated by SDS–PAGE and electrotransferred to nitrocellulose membranes (Ref. 10 401180, Schleicher & Schuell, Dassel, Germany). Membranes were incubated for 1 hr at room temperature in PBS–0.1%

Tween 20–5% skimmed milk (PBST-milk) and then overnight at room temperature with a sheep Nef antiserum in PBST-milk. After three washes with PBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated antisheep in PBST-milk for 1 hr at room temperature. Membranes were washed six times in PBST and once in PBS before being visualized with a chemiluminescent HRP substrate (Ref. 34080, Pierce, Rockford, USA).

Yeast two-hybrid assay

HIV genes were amplified by PCR from the pNL4-3 vector template using the Advantage KlenTaq polymerase (Clontech, Becton Dickinson), with 20 nM of primers incorporating an *Eco*RI or *Xho*I restriction site (Table 1). The PCR products were digested by *Eco*RI and *Xho*I and cloned into the pEG202 "bait" vector (HIS3) to generate fusion proteins with the LexA DNA binding domain. The PCR products for Nef (the full-length Nef, amino acids 1–206, and the processed isoform of Nef, amino acids 58–206) were digested by *Eco*RI and cloned into the pJG4-5 "prey" vector (TRP1) to generate fusion proteins with the activation domain and a hemagglutinin (HA) tag. Nef variants carrying mutations in some important motifs, LL^{165}AA, P$^{75/75}$A, and ΔE$^{62–65}$ (kindly provided by Dr. John Guatelli) were similarly cloned into pJG4-5.

The haploid RFY206 yeast strain (a, *his3 leu2 ura3 trp1 lys2*) was transformed with the bait constructs and the haploid EGY48 yeast strain (α, *trp1 ura3 leu2*::plexop6-LEU2) containing the *lacZ* reporter gene under the control of the LexA promoter on a URA3 plasmid was transformed with the prey constructs. To verify the expression of the cloned HIV gene products, yeast protein extracts were analyzed by Western blot using appropriate antibodies as previously described.[47] Two-hybrid interactions were examined by mating transformed RFY206 yeasts (baits) with transformed EGY48 yeasts (preys) followed by replica plating of the diploids on X-Gal indicator plates containing galactose as described.[48] The empty vectors pEG202 and pJG4-5 were used as a negative control and the interaction between yeast Gle1p bait fusion and Rip1p prey fusion as a positive control for the yeast two-hybrid system.[49]

In vitro binding

Fragments encoding full-length Nef (amino acids 1–206) or cleaved Nef isoform (amino acids 58–206) amplified by PCR (Table 1) were cloned in the pGEX-4T-1 vector (Amersham Pharmacia) as GST-fusion proteins and transformed into *E. coli* HB101. GST fusion protein synthesis was induced by adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3–4 hr. Bacteria were pelleted and resuspended in 10 ml cold PBS–1% Triton X-100, before being disrupted by sonication. The purification of GST fusion proteins was performed using glutathione-Sepharose 4B beads (Amersham Pharmacia) according to the manufacturer's instructions.

HIV genes amplified by PCR (Table 1) were cloned into pcDNA3 or pSP65 vectors under the control of the T7 or Sp6 RNA polymerase promoter and proteins were produced *in vitro* by a coupled transcription-translation system (TnT, Promega) according to the manufacturer's instructions, in the presence of [^{35}S]methionine.

In vitro produced radiolabeled HIV proteins (one-tenth vol of the TnT reaction) were incubated with 40 μg of GST fusion proteins on glutathione-Sepharose beads in 500 μl of binding buffer [0.05% (w/v) Triton X-100, 50 mM HEPES, pH 7.3, 0.1 mM CaCl$_2$, 2 mM MgCl$_2$, 100 mM KCl, 50 μM dithiothreitol, 10% (w/v) glycerol, 0.1% bovine serum albumin] for 2 hr at room temperature on a rotating wheel. After three washes with binding buffer, GST fusion and bound proteins were eluted in 2$\times$ Gel Sample Buffer for 10 min at 95°C and separated by SDS–PAGE. The amount of radiolabeled HIV protein bound to the GST fusion proteins was determined using the Instant Imager system (Packard).

TABLE 1. PRIMERS (5′ → 3′) USED FOR PCR AMPLIFICATION OF THE VARIOUS HIV GENES

Target gene[a]	Forward primer[b]	Reverse primer[c]
Gag55	agggaattcatgggtgcgagagcgtcg	gccccctactcgagttgtgacgaggg
MA	agggaattcatgggtgcgagagcgtcg	ggaggttcgcacctcgaggtaattttggctgac
CA	caggtcagccaagaattcctatagtgcagaac	cttggctcatgcctcgagcaaaactcttgc
NC	caaatccagctaccgaatcatacagaaaggc	ccagatcttccctcgagattagcctgtctctc
p6	ggaaggccagggggaattccttcagagcagacc	gccccctactcgagttgtgacgaggg
PR	ggaactgtatcctttgaatccctcagatcactc	ctcaataggactctcgagaaaatttaaagtgc
RT66	ctttagaattccccattagtcctattgag	ctattccctcgagaaatagtactttcct
RT51	ctttagaattccccattagtcctattgag	ggctgccccatcctcgaggaaagtttctgc
IN	ggaatcaggaaagaattctttttagatgg	ctaatcttttccatctcgagatcctcatcctg
Vif	gcaaagatcatcagaattcatggaaaacagatgg	gttcctctaaaactcgagtgtccattcattg
Vpr	ggaaactgacagagaattcatggaacaagac	gcttccagggctctctcgagggatctactggc
Vpu	gcagtaagtagtagaattcatgcaacctataatag	ccacaattttctgtactcgagcagatcatcaatatcc
gp41	gtggtgcagagagaattcagagcagtggg	cacttgccaccctcgagtagcaaaatc
Nef 1–206	gaaaggattttgcgaattcatgggtggcaagtgg	cctgtagcaagcgaattcagcagttcttg
Nef 58–206	ccttgtagcaagcgaattctcagcagttcttg	cctgtagcaagcgaattctcagcagttcttg

^{a}Amplification of HIV genes according to pNL4-3 sequence (GenBank M19921).
^{b}Underlined letters denote the *Eco*RI site introduced for cloning.
^{c}Underlined letters denote the *Xho*I site introduced for cloning, except for Nef where *Eco*RI was used.

RESULTS

Nef is coimmunoprecipitated by anti-IN, anti-RT, and anti-PR antibodies

Assessment of Nef interaction with other virion proteins was initiated by demonstrating the presence of Nef among proteins immunoprecipitated with antibodies against IN, RT, PR, Vpr, and MA. Virions produced in CEM-GFP cells were lysed, immunoprecipitated by various antisera, and immunoblotted with anti-Nef (Fig. 1). Cell lysate (CL) and viral lysate (VL, 1 μg of p24 equivalent) were used as controls for the detection of Nef. In cell lysates, Nef was predominantly present as a full-length 27-kDa isoform (Fig. 1A, CL). In contrast, in viral lysates, Nef was predominantly present as the cleaved 20-kDa isoform (Fig. 1A, VL).

The immunoprecipitation of wt viral lysates by anti-IN, anti-RT, and anti-PR led to the coimmunoprecipitation of Nef (Fig. 1B). In addition, in contrast to anti-Nef immunoprecipitates, the viral enzymes (IN, RT, and PR) seemed to preferentially coimmunoprecipitate with the processed 20-kDa nef isoform (Fig. 1B). As Nef is processed only in viral particles by the protease, and not in cells, the absence of the Nef p27 isoform excluded a contamination of the viral lysates by microvesicles of cellular origin. In contrast, Nef was not observed in immunoprecipitates using anti-Vpr or anti-MA antisera (Fig. 1B).

To test whether the presence of Nef 20 kDa in immunoprecipitates specifically depended on the presence of viral enzymes, we used clones carrying deletions of RT and IN, or PR, respectively. In the absence of the processed forms of RT and IN, Nef p20 could not be coimmunoprecipitated with the viral enzymes (Fig. 1C), thus excluding that Nef was detected in these immunoprecipitates due to cross-reacting antibodies or incomplete lysis of viral particles. These same blots were reprobed using an anti-HIV polyclonal serum to show that the amount of viral lysate subjected to immunoprecipitation was similar and that the cognate proteins to the immunoprecipitating antiserum were indeed selectively precipitated along with Nef (data not shown).

Nef interacts with IN, PR, and Vpr in the yeast two-hybrid system

We further analyzed Nef interactions using the yeast two-hybrid system. We expressed various HIV proteins as baits, as fusions with the LexA DNA binding domain. Similarly, we expressed Nef as the full-length 27-kDa isoform (Nef 1–206) and as the cleaved 20-kDa isoform (Nef 58–206) as preys fused to the activation domain. The expression of the fusion proteins was verified by immunoblotting with antibodies specific for HIV-1 proteins (not shown), the LexA protein (bait fusion proteins), or the HA tag (prey fusion proteins) (see for instance Fig. 2A).

The integrase bait protein (Fig. 2B; IN) induced β-galactosidase expression only in the presence of Nef prey. In addition, the interaction between integrase and Nef appeared stronger in the presence of the 20-kDa Nef isoform (Nef 58–206). Similarly, the Vpr bait protein (Fig. 2B; Vpr) induced a strong positive interaction in the presence of Nef in this system.
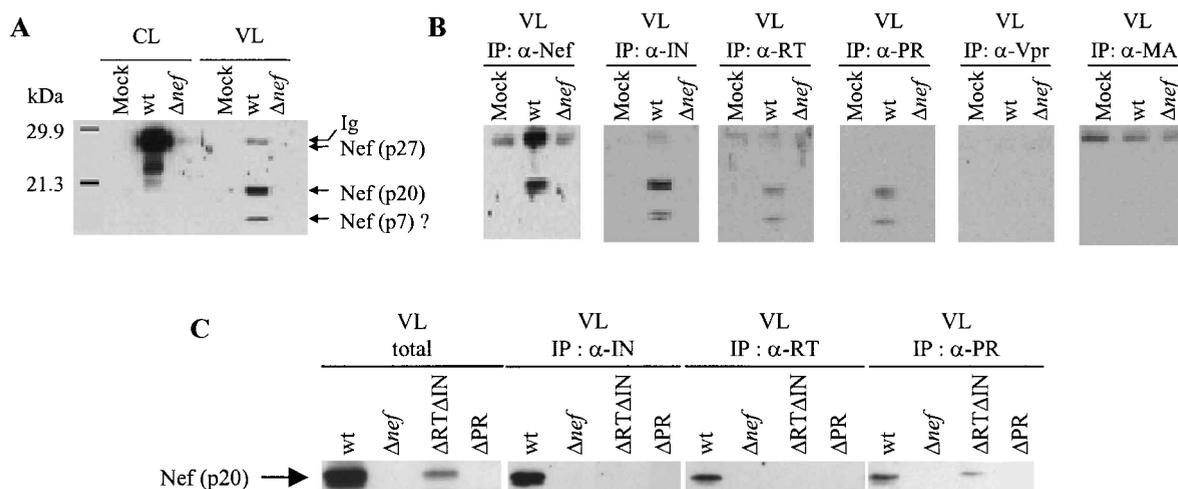

FIG. 1. Immunoblots for Nef. **(A,B)** CEM-GFP cells were transfected with pUC (Mock), pNL43 (wt), or pNL43Δ*nef* (Δ*nef*) and propagated for 2 weeks. Virus containing supernatants were harvested at Days 7, 9, 11, and 13, pooled and pelleted by ultracentrifugation. Cells (harvested on Day 13) (CL) and viruses (VL) were lysed with RIPA buffer. **(A)** Cell and viral lysates without immunoprecipitation. **(B)** Viral lysates (equivalent to 15 μg p24) were immunoprecipitated with viral antisera coupled to protein A-Sepharose: anti-Nef, anti-IN, anti-RT, anti-PR, anti-Vpr, and anti-MA. **(C)** 293T cells were transfected with pNL43 (wt), pNL43Δ*nef* (Δ*nef*), pNL43ΔRTΔIN (ΔRTΔIN), or pNL43ΔPR (ΔPR) and virus containing supernatants were harvested and immunoprecipitated as described above. All samples were separated on a 15% SDS–PAA gel, transferred to a nitrocellulsoe membrane, and immunoblotted for Nef. Ig, contaminating immunoglobulins, immunoglobulins used for immunoprecipitation are recognized by the secondary HRP-conjugated antibodies used for immunoblotting. Arrows indicate the identity of Nef isoforms, the full-length isoform (p27), the processed isoform (p20), and probably the N-terminal anchor of Nef (p7). IP, immunoprecipitation.
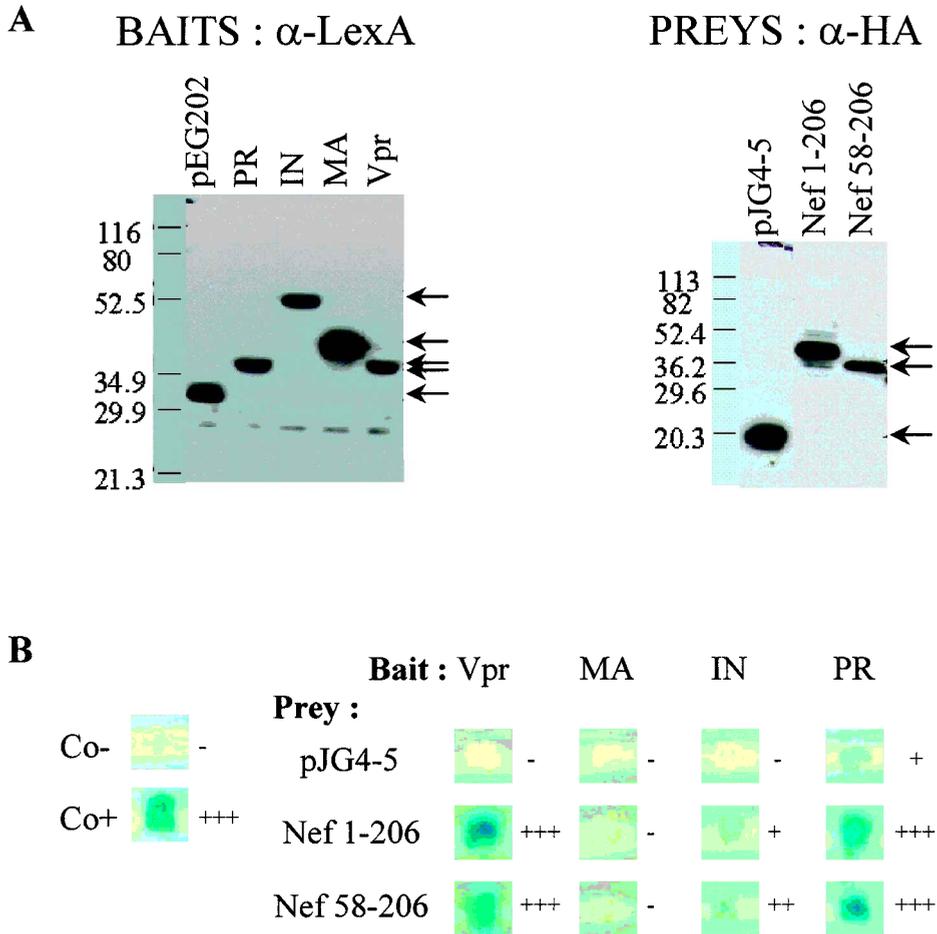
FIG. 2. Yeast two-hybrid screening for viral proteins interacting with Nef. **(A)** Western blots demonstrating the expression of the viral fusion proteins. Bait proteins (pEG202-based) containing the LexA DNA binding domain fused to the viral proteins were expressed in RFY206 yeast strain and prey proteins (pJG4-5-based) containing the activation domain and an HA tag fused to the viral proteins were expressed in the presence of galactose in EGY48 yeast strain. Yeast crude lysates were analyzed by 8% and 15% SDS–PAA gel electrophoresis, respectively. After transfer to a nitrocellulose membrane, immunoblottings were performed using anti-LexA and anti-HA antisera, respectively. Arrows indicate LexA DNA binding domain fused bait proteins and the activation domain-HA-prey proteins, respectively. **(B)** Example of diploid yeasts grown in triple selective medium containing galactose to induce the expression of the prey fusion proteins. The negative control (Co−) contained empty pEG202 and pJG4-5 vectors. The positive control (Co+) contained pEG202-GLE1p and pJG4-5-RIP1p, encoding two yeast fusion proteins known to interact.[49] Diploid yeasts containing Vpr, MA, IN, or PR as bait proteins, and Nef 1–206 or Nef 58–206 as prey proteins are shown. The strength of the reporter signal is expressed in a semiquantitative fashion (− to +++).

The protease bait protein (Fig. 2B; PR) transactivated slightly the β-galactosidase promoter in a prey-independent manner. However, the β-galactosidase signal increased dramatically when Nef expression (as prey protein) was induced, suggesting a direct interaction between Nef and protease.

No interaction was detected between the Nef prey protein and the polyprotein Gag55, MA, CA, NC, p6, RT66, RT51, Vif, Vpu, and gp41 bait fusions (Table 2). Therefore, in this yeast two-hybrid system, Nef (as a prey fusion protein) was able to interact with bait fusion proteins containing IN, PR, and Vpr. Nef mutations in the LL[165] motif [involved in adaptor protein (AP) complex recruitment],[50–51] the polyproline motif P[72]xxP[75] (involved in binding to SH3-protein kinases),[31,52] and the acidic motif EEEE[65] (involved in PACS-1 binding)[53] did not affect Nef binding to IN, PR, and Vpr (data not shown). To map the regions of Nef involved in these interactions, we constructed Nef deletion mutants and tested them for their interaction with IN, PR, and Vpr, but we could not identify any motif responsible for these interactions (data not shown). Nevertheless, removing any of the four α-helices of Nef found in the regions of amino acids 78–118 and 189–206 abolished the interaction with IN, consistent with the hypothesis that Nef tertiary structure is essential for this binding.

Nef interacts with IN and RT in an in vitro binding assay

To assess the specificity of the interactions between Nef and the HIV proteins, in particular with IN, PR, and Vpr, we ex-

TABLE 2. SUMMARY OF NEF INTERACTIONS[a]

		Yeast two-hybrid[b]		In vitro binding[b]	
Nef partners	IP	Nef1	Nef58	Nef1	Nef58
Gag55	NT	−	−	−	−
MA	−	−	−	−	−
CA	NT	−	−	−	−
NC	NT	−	−	−	−
p6	NT	−	−	NT[c]	NT[c]
PR	+	+++	+++	−	−
IN	+	+	++	+	++
RT	+	−	−	+	++
Vif	NT	−	−	−	−
Vpr	−	+++	+++	NA[d]	NA[d]
Vpu	NT	−	−	−	−
gp41	NT	−	−	−	−

[a]−, no interaction detected; +, interaction detected.

[b]The number of + signs correlates with the strength of the interaction.

[c]Not tested due to inability to label with ^{35}S due to the absence of Met and Cys.

[d]Not available due to inability to produce intact protein *in vitro*.

pressed Nef as GST fusion proteins in bacteria. In parallel, the viral proteins (Gag55, MA, CA, NC, PR, IN, RT66, RT51, Vif, Vpr, Vpu, and gp41) were produced *in vitro* by TnT, except for Vpr as we were not able to produce it in our system. GST-Nef

1–206 was able to bind a substantial fraction of IN and the two RT subunits. An even larger fraction of IN, RT66 and RT51 was pulled down by GST-Nef 58–206 (Fig. 3). The binding of IN to GST-Nef was 2.5 times stronger when Nef was present as the cleaved isoform (Nef 58–206) as compared to Nef 1–206. As a positive control for IN, which is known to multimerize,[54–56] we performed *in vitro* binding between GST-IN and TnT-produced IN, which revealed a specific binding that was 5.5 times higher than background (data not shown).

Similarly, GST-Nef was able to pull down specifically RT66 and RT51 (Fig. 3). Again, the binding of RT to GST-Nef was stronger when Nef was present as the cleaved 58–206 isoform compared to Nef 1–206. As a positive control, we assessed *in vitro* binding of GST-RT51 to TnT-produced RT66 that showed a binding that was 3.5 times higher than background (data not shown).[57]

Altogether these results show that IN, RT66, and RT51 bound specifically to full-length Nef and with an even higher affinity to the cleaved Nef isoform (Nef 58–206). In contrast, GST-Nef did not bind specifically Gag55, MA, CA, NC, PR, Vif, Vpu, or gp41 in this assay (Table 2).

DISCUSSION

In this study we present evidence from three approaches to demonstrate binding interactions between Nef and virion proteins. On one hand, using viral particle lysates, Nef was indeed found to coimmunoprecipitate with viral enzymes (IN, RT, and PR). On the other hand, proteins produced in recombinant sys-
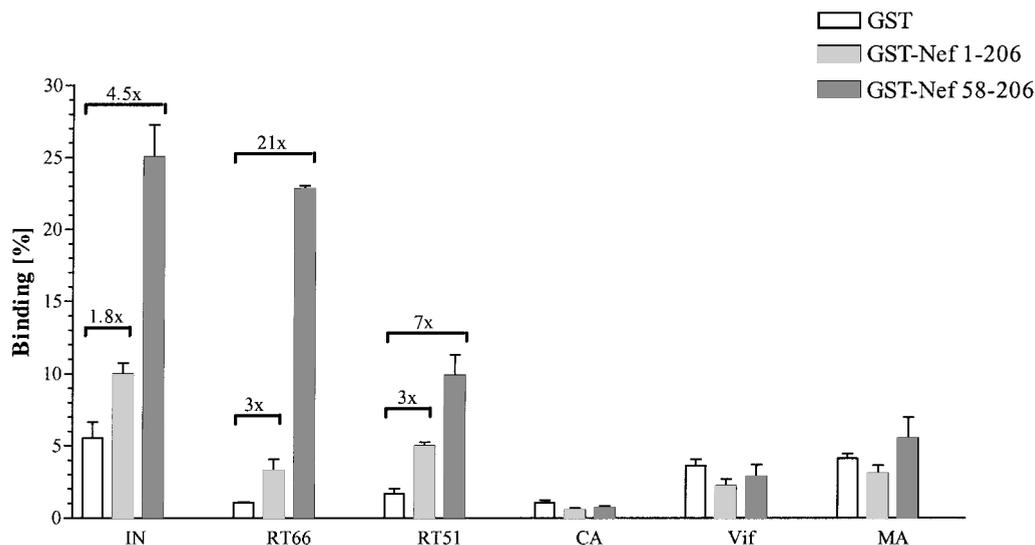

FIG. 3. *In vitro* binding. The two isoforms of Nef (Nef 1–206 and Nef 58–506) were expressed in bacteria as GST-fusion proteins. The GST protein alone was used as a control. One-tenth volume of the TnT reaction product (Gag, MA, CA, NC, PR, IN, RT66, RT51, Vif, Vpu, and gp41) was loaded as the total input. The same amount (one-tenth) of the TnT produced viral proteins was allowed to bind to 40 μg GST, GST-Nef 1–206, or GST-Nef 58–206 fusion proteins on glutathione Sepharose beads. The complexes were then eluted and separated on a 15% SDS–PAA gel. Gels were then dried and exposed for autoradiography. The binding of each viral protein produced by TnT with the GST-fusion proteins was quantified by PhosphorImager and plotted as a fraction (%) of the total input bound to the GST-fusion proteins. The percentage binding of *in vitro* produced IN, RT66, RT51, CA, Vif, and MA with GST alone (white bars), GST fused to Nef full length (GST-Nef 1–206, light gray bars), and GST fused to the processed Nef isoform (GST-Nef 58–206, dark gray bars) are shown (results from triplicate experiments). The ratios in binding of Nef 1–206 and Nef 58–206 compared to background (represented by the nonspecific binding of *in vitro* produced proteins to GST-glutathione Sepharose alone) are indicated.

tems, namely the yeast two-hybrid system and a GST pull-down assay, were used. The yeast two-hybrid system showed that IN, PR, and Vpr were candidates for a direct interaction with Nef, and the GST pull-down assay showed that IN and RT were binding to Nef. The results of these assays are summarized in Table 2. Assay characteristics and differences in protein conformation may explain discrepancies in the data generated by the different assays.

The viral integrase appears as the best candidate for interacting directly with Nef, as a binding interaction was detected in all three assays. The interaction between Nef and IN was stronger when Nef was present as the cleaved isoform (Nef 58–206), both in the yeast two-hybrid and the GST pull-down assays. Based on Nef protein truncations in the yeast two-hybrid assay, the Nef tertiary structure seemed to be essential for its interaction with IN, as removing any of the α-helices abolished this interaction. However, a panel of deletion mutants did not allow us to refine the mapping of the Nef integrase interacting domain.

In addition to coimmunoprecipitating in virion lysates using anti-RT antibodies, Nef was found to interact with the two reverse transcriptase subunits RT66 and RT51 in the GST pull-down assay. As for IN, the cleaved Nef isoform (Nef 58–206) fused with GST pulled down more efficiently the RT subunits than the full-length Nef (Nef 1–206). In contrast, the interaction between Nef and RT subunits was not observed in the yeast two-hybrid assay. However, although RT66 and RT51 are known to heterodimerize,[57] RT66 and RT51 as prey and bait, respectively, also did not interact in this assay, suggesting that in these conditions, RT did not adopt a conformation allowing the expression of its binding characteristics.

Nef is processed by the viral protease in the viral particle.[37] It comes therefore as no surprise that Nef, as a bait fusion protein, tested positive for interacting with PR prey in the yeast two-hybrid assay. However, an attempt to map the Nef domain responsible for this binding using a panel of deletion mutants did not allow us to identify domains responsible for PR binding. Therefore, we could not confirm the specificity of this positive reaction. In addition, Nef-GST did not pull down *in vitro*-transcribed PR.

While Nef did not coimmunoprecipitate with Vpr, this latter protein interacted with Nef in the yeast two-hybrid system. However, as for PR, we could not confirm the specificity of this positive reaction, neither by using Nef deletion mutants in the yeast two-hybrid (data not shown) nor by *in vitro* binding as Vpr could not be produced *in vitro*.

Nef is a substrate for the viral protease. As a virion protein, Nef has been demonstrated to be partially processed by proteolytic cleavage at amino acid 58, generating a 20-kDa fragment[35,37–39] carrying the majority of functional domains and the organized core of Nef,[58] as well as a 7-kDa fragment corresponding to a disorganized NH_2 terminal arm. At the present time, studies of cleavage site mutants have not revealed any functional correlate for this proteolytic event.[41,42]

Here, we identify the processed isoform Nef 58–206 as a partner with a higher affinity for IN and RT than the full-length Nef. It has been proposed that the N-terminal arm of Nef (Nef 1–70) could cover a hydrophobic region in the Nef folded core domain (Nef 71–148 and Nef 178–203).[9,59] Thus, it is possible that removing this N-terminal arm of Nef, as is the case with the Nef 58–206 isoform, would expose this hydrophobic area, which may interact with hydrophobic domains in partners and increase binding affinity. By enhancing Nef affinity for viral enzymes, the proteolytic processing might favor the recruitment of the 20-kDa Nef fragment into viral cores.

Nef, mostly as a processed isoform, is present in viral cores[36] and is associated with the viral ribonucleoprotein complex.[60] Our data, using three different approaches, demonstrate that Nef potentially interacts, not only as a recombinant protein *in vitro* or in yeast, but also in viral particles with viral core proteins. We hypothesize that this interaction is the molecular basis for the presence of Nef in HIV cores, probably through a direct interaction with IN and perhaps RT.

The binding of Nef to viral enzymes suggests that Nef may accompany the viral genome through the reverse transcription, nuclear translocation, and integration steps, and opens new possibilities for Nef to affect infectivity. First, by interacting with IN and RT, Nef may affect the enzymatic activity of these enzymes *in vivo*. Second, by recruiting kinases, Nef has been hypothesized to induce the phosphorylation of virion components in the producer cell.[61,62] The ability of Nef to interact with various kinases[21] might also induce the recruitment of kinases to the reverse transcription complex (RTC) or preintegration complex (PIC) in the target cell, where it could modify the complex components. Finally, Nef has the potential to interact with cellular sorting and transport machineries.[9,10,63] Nef may then contribute to direct the preintegration complex from the cytoplasm to the perinuclear region to favor its nuclear translocation. Indeed, Nef has been shown to transiently localize in the nucleus in acute and chronic infections.[64–66] Alternatively, Nef, by binding to viral enzymes or Vpr, could affect the stability of the RTC,[67–69] thus preventing RTC degradation or disassembly, resulting in a more efficient viral DNA synthesis.

While abundant data regarding interactions between Nef and cellular proteins have been reported,[9] the present study is the first description of an interaction between Nef and viral proteins in the context of viral particles that may explain its localization in cores. In addition, no phenotype has been attributed to the cleaved Nef isoform to date. Our data show for the first time a biochemical difference between Nef and processed Nef, in that the cleaved Nef isoform bound to viral enzymes more efficiently than full-length Nef. The functional significance of this association remains to be defined.

ACKNOWLEDGMENTS

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