Comparative Proteomic Analysis of HIV-1 Particles Reveals a Role for Ezrin and EHD4 in the Nef-Dependent Increase of Virus Infectivity

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Nef is a human immunodeficiency virus type 1 (HIV-1) auxiliary protein that plays an important role in virus replication and the onset of acquired immunodeficiency. Although known functions of Nef might explain its contribution to HIV-1-associated pathogenesis, how Nef increases virus infectivity is still an open question. *In vitro*, Nef-deleted viruses have a defect that prevents efficient completion of early steps of replication. We have previously shown that this restriction is not due to the absence of Nef in viral particles. Rather, a loss of function in virus-producing cells accounts for the lower infectivity of *nef*-deleted viruses compared to wild-type (WT) viruses. Here we used DiGE and iTRAQ to identify differences between the proteomes of WT and *nef*-deleted viruses. We observe that glucosidase II is enriched in WT virions, whereas Ezrin, ALG-2, CD81, and EHD4 are enriched in *nef*-deleted virions. Functional analysis shows that glucosidase II, ALG-2, and CD81 have no or only Nef-independent effect on infectivity. In contrast, Ezrin and EHD4 are involved in the ability of Nef to increase virus infectivity (referred to thereafter as Nef potency). Indeed, simultaneous Ezrin and EHD4 depletion in SupT1 and 293T virus-producing cells result in an ~30 and ~70% decrease of Nef potency, respectively. Finally, while Ezrin behaves as an inhibitory factor counteracted by Nef, EHD4 should be considered as a cofactors required by Nef to increase virus infectivity.

In addition to the *gag pol* and *env* genes that encode structural elements common to all retroviruses, human (HIV) and simian (SIV) immunodeficiency viruses have acquired auxiliary genes—*vif*, *vpr*, *vpu*, *vpx*, and *nef*—in the absence of which virus replication *in vitro* is possible in some, but not all experimental systems. Such nonpermissive systems have revealed that most auxiliary proteins favor virus replication by promoting the degradation of specific cellular factors (1–6).

Nef plays a major role in the pathogenesis associated with HIV and SIV infection of humans and macaques, respectively. Viruses harboring mutations that abrogate the expression of Nef have been shown to replicate poorly *in vivo*, leading to no or delayed immunodeficiency syndromes (7, 8). Recently, SIV Nef has been shown to favor virus release by counteracting BST2/Tetherin (3, 9), which helps to explain why *nef*-deleted virions are less pathogenic than their wild-type (WT) counterparts (7). However, the fact that not all SIV Nef alleles and no HIV Nef allele counteract BST-2/Tetherin strongly suggests that another property common to HIV and SIV Nef is also involved in its ability to favor viral replication in primates. Although it is not clear how Nef makes HIV and SIV more pathogenic than *nef*-deleted viruses, known functions of Nef characterized *in vitro* likely contribute to increase the efficiency of viral replication in the host (for reviews, see references 10, 11, and 12). First, Nef has been shown to alter the trafficking of many host proteins in infected cells by interfering with the endosomal network. The downregulation of the cell surface levels of CD4, CXCR4, and CCR5 (5, 13), which serve as HIV receptors (14–16), is thought to prevent cytotoxic superinfection, while selective major histocompatibility complex class I (MHC-I) downregulation (17, 18) allows immune evasion, which favors virus dissemination. Second, Nef reprograms host-cell signaling networks in favor of viral gene expression (19). Third, a direct

effect of Nef on the actin cytoskeleton was proposed to facilitate viral egress and cell-to-cell virus spread (20, 21).

Another aspect of Nef that might directly impact HIV and SIV associated pathogenesis is evidenced in cell-free single-round infection-competent viruses where WT viruses are consistently 5- to 20-fold more infectious than *nef*-deleted viruses (22, 23). Unlike the other functions of Nef, which underlying mechanisms have been partially deciphered, the ability of Nef to increase virus infectivity remains obscure. An effect of Nef on fusion has been suggested based on the ability of Nef to modulate the incorporation of HIV-1 envelope glycoproteins (Env) into the viral membrane through its CD4 downregulation activity in virus-producing cells (24, 25). A CD4 downregulation-independent effect on fusion has also been suggested (26). Recently, a direct link between Nef and Env, independently of CD4 has been demonstrated by Lai et al., who reported that the HIV-1_{JR-FL} Env is not sensitive to the effect of Nef, suggesting a link between Nef potency and fusion (27). On the contrary, other reports have ruled out an effect of Nef on the fusion process (28–30; our unpublished data), supporting that the advantage of WT viruses over *nef*-deleted viruses mani-

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fects itself in postentry steps, which is in agreement with early data describing an effect of Nef at the level of reverse transcription (31–33). Compelling data also came from the analysis of the effect of Nef on pseudotyped viruses infectivity. While Nef increases the infectivity of virions pseudotyped with envelope glycoproteins that promote fusion at neutral pH, both WT and *nef*-deleted virions pseudotyped with vesicular stomatitis virus (VSV) or subgroup A Rous sarcoma virus (RSV-A) envelope glycoproteins, which fusion activity requires virion endocytosis and endosome acidification, are equally infectious (34, 35). Based on this dichotomy and the postfusion inhibitory effect of the target-cell actin network on HIV-1 Env-mediated entry, it has been hypothesized that Nef might help incoming capsids go through the actin network located at the inner face of the plasma membrane (36). This model has been challenged recently by Melikyan and coworkers, who have demonstrated that HIV Env-mediated fusion takes place in early endosomes after virus endocytosis (37, 38). According to these results, virions would go through the cortical actin network in endocytic vesicles prior to membrane fusion in early endosomes and virus capsid delivery into the cytoplasm. Furthermore, Pizzato et al. have demonstrated that Tva pseudotyped virus were sensitive to the effect of Nef, despite entry into RSV-A envelope glycoprotein-expressing cells being mediated by virus endocytosis and fusion taking place in acidic endosomes (35). Together, these studies suggest that in addition to a possible effect of Nef at the cortical actin level, Nef also promotes the completion of postfusion steps that take place after the virus has gone through this actin network. It has been speculated that virus-borne Nef molecules might play a direct role in such compartments; however, no correlation has been found between the incorporation of Nef into virions and its ability to increase virus infectivity (39–41). In fact, Nef might leave another kind of imprint on WT virions in the course of their biogenesis that eventually improves the completion of postentry steps. In agreement with this hypothesis, Lai et al. have demonstrated that Nef affects the exposure of particular Env epitopes at the surfaces of virions (27). Although this was clearly disconnected from the ability of Nef to increase virus infectivity, it nevertheless confirmed that Nef affects the conformation of virions.

Extensive proteomic analysis has demonstrated the presence of host cell-derived proteins in virions (42–47). Given the ability of Nef to interfere with the endosomal network, we hypothesized that it might modify the virus proteome. In the present study, we compare the protein composition of WT and *nef*-deleted viral particles. Two quantitative methods were used: differential fluorescence two-dimensional (2-D) gel electrophoresis (DIGE) and multiplexed isobaric tagging for relative and absolute quantification (iTRAQ). Our results indicate that while some cellular proteins are enriched in WT virions, others are enriched in *nef*-deleted virions. Among the candidates selected for functional analysis, Ezrin and EHD4 appear to be involved in the ability of Nef to increase virus infectivity.

MATERIALS AND METHODS

Cells and reagents. HEK293T cells were obtained through the American Type Culture Collection and grown in complete medium composed of Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU of penicillin/ml, and 100 µg of streptomycin/ml (Invitrogen, Cergy-Pontoise, France). Alternatively, CD293 and FreeStyle293 (Invitrogen) were substituted for DMEM. HeLa-CD4 were

obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), and grown in complete medium supplemented with G418 (PAA, Austria) at 0.2 mg/ml. SupT1 cells were kindly provided by Massimo Pizzato (University of Trento, Trento, Italy) and grown in RPMI (Invitrogen) supplemented with serum and antibiotics as described above. All cells were grown at 37°C with 5% CO₂.

The pNL4-3, pBR43IeG-*nef*+, and pBR43IeG-*nef*− plasmids used to generate replication-competent viruses were obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, NIH. The *nef*-deleted version of pNL4-3 (pNL4-3 Xho) was generated as described previously (39). Full-length Ezrin, CD81, EHD4, and ALG-2-encoding plasmids were obtained from Monique Arpin (Institut Curie, Paris, France), Eric Rubinstein (Institut André Lwoff, Villejuif, France), Steve Caplan (University of Nebraska, Nebraska Medical Center), and Rémi Sadoul (Institut des Neurosciences, Grenoble, France), respectively. The pSRαNef_{LAI} WT and the Nef XhoI are described elsewhere (39). Small interfering RNA (siRNA) used in the present study (Ezrin, GCGC GGAGCUGUCUAGUGAUU; CD81, GCACCAAGUGCAUCAAGU AUU; EHD4, GACCACCUUCAUCAGAUACUU; and ALG-2, GACAG GAGUGGAGUGAUUUU) were purchased from Eurogentec based on published sequences (48–51).

Protein analysis by Western blotting, followed by immunodetection, was performed as described elsewhere (39), except for the CD81 resolution that was carried out under nonreducing conditions and Ezrin immunodetection that required 3% bovine serum albumin substitution for 5% nonfat dry milk. The CD81 specific antibody was a gift from E. Rubinstein and was used at a 1:2,000 working dilution. EHD4-specific antibodies were from S. Caplan and also purchased from GeneTex (Euromedex, France) and diluted 1:500 and 1:100, respectively. Ezrin-specific antibodies were from Paul Mangeat (Centre de Recherche de Biochimie Macromoléculaire, Montpellier, France) and also purchased from Epitomix (Euromedex) and diluted 1:10,000 and 1:1,000, respectively. HIV Nef (52) and p24 antibodies were obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, NIAID, NIH (working dilutions, 1:1,000 and 1:10,000, respectively). The ALG-2 antibody (working dilution, 1:2,000) was purchased from Swant (Marly, Switzerland). All horseradish peroxidase-coupled secondary antibodies were purchased from Sigma (Saint-Quentin Fallavier, France) and used at a 1:2,000 dilution.

Viral production, ultracentrifugation, and infection. Replication-competent NL4-3 and NL4-3 Xho viruses used for DIGE and iTRAQ were made in 293T cells transfected with the calcium phosphate DNA precipitation technique as described elsewhere with minor modifications (39). Cells were transfected with 15 µg of pNL4-3 or pNL4-3 Xho/T75, and the medium was replaced the next day with that indicated in figure legends. Supernatants were harvested 24 h later and subjected to ultracentrifugation as described elsewhere (39) and in the legend to Fig. 1. Pellets were then resuspended 30 min at 4°C in DIGE buffer [8 M urea, 2 M thiourea, 50 mM dithiothreitol, 4% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}] and centrifuged at 100,000 × *g* for 1 h at 10°C. Supernatants were collected for DIGE or iTRAQ analysis.

Single-round infection-competent viruses carrying the green fluorescent protein (GFP) gene were made in 293T cells transfected with JetPRIME (Ozyme, Saint-Quentin-en-Yvelines, France) as follows. Cells were seeded in six-well plate at a density of $\sim 2 \times 10^5$ cells/well and transfected after 48 h with 2 µg of a DNA mix containing the HIV-1 packaging plasmid (pCMVΔP1ΔenvpA), the GFP-encoding HIV-1 vector (pHIVec2.GFP), the HIV-1_{HXBc2} envelope glycoprotein-encoding plasmids (pSVIIIenv), and either pSRαNef_{LAI} or the Nef XhoI construct at a 3:3:1:1 ratio, respectively. pHCMV-G and a Rev-encoding plasmid (1:1 mix) were substituted for pSVIIIenv to generate VSV-G-pseudotyped virions. The medium was changed 24 h post-transfection, and supernatants were harvested after an additional 24-h

incubation time, centrifuged at $270 \times g$, filtered through 0.45- μm -pore-size membranes, and stored at -80°C . Where indicated, virions were pelleted from the cell culture supernatant. Virus-producing cells were also harvested and solubilized as described elsewhere (39). In order to achieve candidate protein overexpression in virus-producing cells, specific plasmids were added to the transfection mix in the same proportion as the Nef (or Nef Xho) construct. In the series of experiments that required candidate protein silencing, the cells were seeded and transfected 24 h later with specified siRNAs and 24 h later as described above with viral plasmids. The virus concentration in cell culture supernatant was measured with an Alliance HIV-1 p24 ELISA kit (Perkin-Elmer, Courtaboeuf, France), and the virus infectivity was measured on HeLa-CD4 cells as described previously (39).

For single-round entry analysis of viruses produced in SupT1 cells, as well as spreading infection experiments in SupT1 cells, replication-competent WT and *nef*-deleted GFP-encoding viruses were produced in 293T cells cotransfected as described earlier (39) with 15 μg of either pBR43IeG-nef+ or pBR43IeG-nef- (53, 54), respectively, and 1 μg of pHCMV-G. VSV-G pseudotyping ensured similar rates of infections for all SupT1 cell lines. Infectivity analysis of viruses generated from SupT1 cells was conducted as described elsewhere (55). Briefly, VSV-G-pseudotyped viruses were concentrated by ultracentrifugation and resuspended in complete medium. Viral stocks were normalized for p24 content and incubated onto SupT1 cells. The cells were washed extensively at 12 h postinfection, the medium was replaced 24 h later, and the viruses were harvested after an additional 24 h of incubation time. Virus infectivity was then assayed on HeLa-CD4 cells as described previously (39) with minor modifications. The medium was changed at 6 h postinfection, and cells were grown in complete medium supplemented with zidovudine at 10 μM in order to prevent spreading infection. GFP expression was measured by flow cytometry at 60 h postinfection.

Spreading infection was conducted as follows. Target SupT1 cells ($\sim 4 \times 10^6$ in 4 ml of complete RPMI) were infected with 10 ng of p24 of VSV-G-pseudotyped viruses. After a 12-h incubation period, the cells were washed extensively with phosphate-buffered saline (PBS) and seeded at a concentration of 0.5×10^6 cells/ml. The cells were diluted 1:3 every 2 to 3 days and analyzed by flow cytometry to record the percentages of GFP-positive events throughout the infection time course.

Ezrin and EHD4 silencing in SupT1 cell lines. Ezrin and EHD4 shRNA were generated from lentiviral pLKO.1 vectors purchased from Thermo Scientific (ABgene, Epsom, United Kingdom). The mature shRNA sense sequences were as follows: Ezrin, CGTGGGATGCTCAAA GATAAT, and EHD4, CAGTCGCTTTGGAAACGCTTT. VSV-G-pseudotyped shRNA viruses specific for Ezrin and EHD4 were made in 293T cells transfected by the calcium phosphate DNA precipitation technique as described above by substituting the pHlvec2.GFP plasmid with the appropriate pLKO.1 plasmid. SupT1 cells were transduced with lentiviral vectors, either separately or in combination, and selected 72 h later in RPMI (Invitrogen) supplemented with 10% FCS, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 1 μg of puromycin/ml (Life Technologies). After selection, the cells were propagated in the absence of puromycin and regularly checked for protein silencing.

DIGE and iTRAQ. DIGE was performed according to procedures described elsewhere with minor modifications (56). Proteins from three independent WT (W1, W2, and W3) and three independent *nef*-deleted mutant (M1, M2, and M3) virus samples were labeled with either Cy3 or Cy5. A mix of equal amounts of all samples was labeled with Cy2 and constituted an internal standard (IS). Portions (50 μg) of each sample (W, M, and IS) were combined as follows and resolved on three gels: W1-Cy5/M1-Cy3/IS-Cy2, W2-Cy3/M2-Cy5/IS-Cy2, and W3-Cy5/M3-Cy3/IS-Cy2. Sample resolution was performed by isoelectrofocusing in the first dimension on 18-cm pH 3–11 IPG strips (GE Healthcare) and on 8 to 18% SDS-PAGE gels in the second dimension. Cy2, Cy3, and Cy5 components were individually imaged as described previously (56). Relative quantification of spot intensity and statistical evaluation were carried out with

DeCyder software 6.5 (GE Healthcare). The results for each spot intensity were normalized to the IS and compared across all gels. Relative incorporation ratios (*nef*-deleted/WT) of cell-derived proteins and statistical data for each differentially expressed spot were obtained. Portions (25 μg) of proteins from each of the W1-3 and M1-3 unlabeled samples were also mixed and resolved by a 2-D gel. Proteins were then stained with mass spectrometry (MS)-compatible Coomassie blue reagent, the image was matched to the DIGE acquisition, and spots of interest were manually excised for protein identification by mass spectrometry (56).

iTRAQ analysis was performed on samples prepared independently from those used in DIGE, as described elsewhere (57). Portions (70 μg) of proteins from each WT virus duplicate were reduced, alkylated, digested, and labeled with the 114 or 115 isobaric probe according to the protocol of the iTRAQ reagents kit (AB Sciex, Les Ulis, France). Similarly, *nef*-deleted duplicate samples were labeled with the 116 or 117 probe. Samples were mixed and subjected to strong cation exchange and reversed-phase nanoliquid chromatography prior to tandem MS (MS/MS). Acquisitions were combined, and protein identification and quantification were done with ProteinPilot version 2.0.1 (AB Sciex). The search was performed against the human protein database from the International Protein Index (IPI), version 3.38, concatenated with HIV-1_{NL4-3} proteins and a contaminant protein database. The data were processed as described in reference 57.

RESULTS

Virus production settings. The proteomic analysis was conducted on HIV-1 particles produced in the non-T-cell line HEK293T, allowing the investigation of the infectivity phenotype independently of the effect of Nef on CD4. In this experimental system, Nef induced a 6- to 8-fold increase of virus infectivity, which is comparable to the effect observed when virions are produced in most T-cell lines (58). Virion purification was first optimized to avoid contaminations by cell-derived microvesicles. When cells were grown in complete medium, the material pelleted from supernatants through a single 20% sucrose cushion revealed similar protein patterns whether cells were mock-transfected or transfected with WT pNL4-3 (Fig. 1A). Centrifugation over two sucrose cushions allowed the recovery of samples in which HIV-1 matrix and capsid proteins were unambiguously identified, while higher-molecular-mass virus proteins were still undistinguishable from contaminants (Fig. 1B). Various culture media were tested, of which CD293 was the most satisfactory in terms of virus amount/contaminants ratio (Fig. 1C and D).

Differential gel electrophoresis of WT and *nef*-deleted virions. Three batches of WT and three batches of *nef*-deleted viruses were produced independently and processed as described in Materials and Methods for DIGE analysis. In Fig. 2A, one WT and one *nef*-deleted sample are resolved together with an internal standard (IS). Most proteins were equally abundant in both samples (Fig. 2A, right panel, white spots). Nef, which represents the best internal control (Fig. 2A, spots 1a and 1b, corresponding to full-length and cleaved Nef, respectively, and 2C), gave the highest WT/*nef*-deleted ratio (Fig. 2B, down 17-fold in *nef*-deleted virus). Besides Nef, 19 spots were selected with DeCyder, corresponding to proteins significantly enriched either in the WT or the *nef*-deleted sample (Student *t* test, $P < 0.05$). Of 19 spots, 8 were identified by MS analysis as the δ subunit of the translocon-associated protein (TRAP δ , spot 2) and both α and β chains of glucosidase II (Gluc II, spots 3 and 4). Ezrin-Radixin-Moesin family proteins (ERMs) were also differentially incorporated; however, due to their sequence similarity, their identity could not be ascertained at this stage of the analysis (spots 5a to 5e). Whereas spot 5a represents

FIG 1 Optimization of HIV-1_{NL4-3} particles purification. Mock or pNL4-3 (WT)-transfected cells were incubated 24 h in complete medium (A and B), DMEM, CD293, or Free style 239 medium (C) as indicated. Supernatants were then harvested, cleared by low-speed centrifugation and filtered prior to subsequent purification steps. (A) Supernatants were layered on top of a 20% sucrose cushion and ultracentrifuged. (B and C) Supernatants were layered on top of a discontinuous sucrose gradient composed of 20 and 60% sucrose cushions. After ultracentrifugation, the 20 to 60% interphase was recovered, diluted in PBS, and subjected to an additional ultracentrifugation step. Pellets were resuspended in DIGE buffer, resolved by 12% acrylamide SDS-PAGE, and visualized by silver staining. About 75% of virions released by cells grown in a T75 flask were loaded in each lane. (D) Material from 200 ml (~20 T75 flasks) of mock-, pNL4-3 (WT)-, or pNL4-3 Xho (*nef*-deleted)-transfected 293T cell supernatants were pelleted as described in panel C. Pellets were resuspended in 200 μ l of DIGE buffer, 13 μ l of which was mixed with reducing loading buffer and resolved by 12% acrylamide SDS-PAGE without boiling. The proteins were then visualized by silver staining. Nine samples prepared independently are resolved on this gel.

full-length ERM proteins, spots 5b to 5e likely represent cleaved forms, as already reported (45).

Four-plexed isobaric tagging for relative and absolute quantification applied to WT and *nef*-deleted samples. Two WT samples were prepared as in Fig. 1, and labeled with the 114 or the 115 isobaric probes following tryptic digestion. Similarly, two *nef*-deleted derived samples were labeled with the 116 or 117 probes. The four samples were mixed and subjected to MS/MS analysis. Integration of the 114, 115, 116, and 117 probe signals for each peptide allowed the calculation of the relative abundance of the corresponding protein in the WT and *nef*-deleted samples. About 1,000 cell-derived proteins were identified in viral samples; 421 of these had peptide ratios confidently interpretable (see Table S1 in the supplemental material). Among these proteins, 343 were equally abundant in the two WT samples (114:115 control ratios within a 1.2-fold up or down range [data not shown]). Similarly, 350 proteins were equally abundant in the two *nef*-deleted samples (116:117 control ratios within a 1.2-fold up or down range [data not shown]). Both 114:115 and 116:117 ratios fell within this range for 295 proteins. This group was further screened for candidate proteins, which incorporation index was changing by >1.2-fold in at least three of the four *nef*-deleted/WT experimental ratios (116:114, 116:115, 117:114, and 117:115; Table 1). Glucosidase II α and ERMs found enriched in WT and *nef*-deleted samples by DIGE, respectively, showed a similar incorporation profile by iTRAQ. Glucosidase II β and TRAPs were also identified by iTRAQ (see Table S1 in the supplemental material); however, they did not meet the criteria described above.

In order to check whether identified proteins play a role in the ability of Nef to increase virus infectivity (referred to thereafter as Nef potency), we chose to modulate their expression levels in virus-producing cells and measure WT and *nef*-deleted progeny virus infectivity. Glucosidase II and the ERM family member Ezrin, detected by both proteomic methods, were selected for further characterization. The role of the tetraspanin CD81 was investigated based on previous reports suggesting its effect on HIV-1 replication (59, 60). EHD4, a key regulator of the transport of endocytic vesicles (50), a process in which Nef interferes, and PDCD6/ALG-2, a ligand of AIP-1/Alx involved in virus biogenesis (61, 62), were also chosen. The effects of candidate proteins overexpression or silencing on virus infectivity and Nef potency are summarized in Table 2.

Role of Ezrin and CD81 in the biogenesis of infectious viral particles. Members of the ERM protein family and tetraspanins interfere with the replication of HIV-1 and other viruses (63–67; for a review, see reference 68); however, no connection has been established between Nef and ERMs or tetraspanins. siRNAs were used to decrease the expression levels of CD81 or Ezrin in 293T cells. Cells were subsequently transfected to produce HXBc2 Env-pseudotyped virions in the presence (WT Nef) or in the absence (Nef Xho) of Nef. VSV-G-pseudotyped virions that are insensitive to the effect of Nef were also generated in similar conditions. As shown in Fig. 3A, Gag precursor cleavage intermediates displayed similar patterns and expression levels throughout the experimental settings (Fig. 3A, lower panel). Expression levels of endogenous Ezrin and CD81 in siRNA-treated cells were decreased by ~70% and below the limit of detection, respectively (Fig. 3A, upper and middle panels). Ezrin or CD81 depletion in HXBc2 Env-pseudotyped virion-producing cells resulted in an ~2-fold increase of *nef*-deleted (Nef Xho) virus infectivity, whereas VSV-G-pseudotyped virions were equally infectious regardless of the siRNA treatment (Fig. 3B). As expected, HXBc2-pseudotyped virions made in the presence of WT Nef were ~6-fold more infectious than *nef*-deleted virions (Fig. 3B and Table 2). Of note, Nef potency remained unchanged when virions were produced in cells depleted from CD81 but significantly decreased by 22% when virions were produced in Ezrin-depleted cells (Table 2). This suggests a functional link between Ezrin and the ability of Nef to increase virus infectivity. We next investigated the consequences of CD81 or Ezrin overexpression on HIV-1 infectivity. CD81 overexpression, as documented in Fig. 3C, caused an ~4-fold decrease of virus infectivity (Fig. 3D) and yet had no effect on Nef potency (Fig. 3D and Table 2). Together with the lack of effect on VSV-G-pseudotyped viruses (Fig. 3D), these results mirror the effect of CD81 silencing described above. Interestingly, while Ezrin siRNA increased virus infectivity and decreased Nef potency, neither parameter was affected by Ezrin overexpression (Fig. 3D and Table 2).

Role of EHD4 and ALG-2 in the biogenesis of infectious viral particles. EHD4 is a key regulator of the transport of endocytic vesicles (50), which traffic is perturbed by Nef, while ALG-2 is a ligand of AIP-1/Alx, an ESCRT factor involved in HIV-1 budding (61, 62). This prompted us to investigate the role of EHD4 and ALG-2 in the process of HIV-1 biogenesis. siRNA and cDNA constructs were used to manipulate the expression levels of ALG-2 or EHD4 in virus-producing cells as described in Fig. 3. ALG-2 silencing was close to 80% (Fig. 4A, middle panel), while EHD4 was undetectable upon siRNA treatment (Fig. 4A, upper panel). No

TABLE 1 iTRAQ analysis of WT (114 and 115 probes) and *nef*-deleted (116 and 117 probes) NL4-3 viral particles

N ^a	Prot	Peptide ^c	% Cov ^d	IPI ^e	Name ^f	Control ratio		Experimental ratio				Nef-deleted/WT ratio ^g		
						114:115	116:117	116:114	116:115	117:114	117:115	Ratio	P-Value	EF
68	15.49	7	27.4	00005578	EHD4 EH domain-containing protein 4	1.07	0.96	1.26	1.34	1.33	1.44	1.34	4.8E-15	1.06
801	2	2	9.1	00103887	OR8D1 Olfactory receptor 8D1	1.05	0.89	1.22	1.30	1.38	1.47	1.33	1.8E-04	1.14
244	6	3	10.2	00657752	CD81	0.96	0.93	1.28	1.25	1.41	1.36	1.31	1.9E-19	1.06
462	3.05	4	18.8	00025277	PDCD6 Programmed cell death protein 6 (ALG-2)	1.04	0.89	1.21	1.26	1.37	1.43	1.31	1.8E-04	1.13
12	41.92	14	45.9	00872684	EZR Ezrin	0.98	0.90	1.25	1.25	1.40	1.38	1.30	2.1E-08	1.09
506	2.54	5	18.3	00657663	NBPF20;NBPF10;NBPF11;NBPF15;NBPF8; KIAA1245;NBPF9;NBPF14;NBPF16; NBPF1 Isoform 2 of Neuroblastoma breakpoint family member 10	1.12	0.93	1.15	1.31	1.27	1.44	1.28	1.1E-03	1.15
259	5.31	4	17.6	00646917	NUDT21 Cleavage and polyadenylation specificity factor subunit 5	1.17	0.84	1.07	1.26	1.28	1.50	1.27	1.1E-03	1.13
80	14.14	7	31	00017367	RDX Radixin isoform b	0.95	1.01	1.30	1.25	1.31	1.25	1.26	2.4E-08	1.07
24	30.59	20	42.2	00221226	ANXA6 Annexin A6	1.05	1.01	1.21	1.28	1.20	1.29	1.23	6.1E-08	1.07
97	12.59	8	54.3	00015148	RAP1B Ras-related protein Rap-1b precursor	1.07	0.92	1.12	1.20	1.24	1.31	1.21	4.7E-05	1.09
554	2.32	4	13	00413780	PKN3 Serine/threonine-protein kinase N3	0.99	1.04	1.26	1.25	1.21	1.15	1.21	7.0E-03	1.14
58	16.69	12	46.2	00872387	ENSP00000381447 (Fragment)	0.99	1.00	0.82	0.82	0.83	0.83	0.81	7.7E-08	1.07
149	8.72	3	40.8	00003377	SFRS7 Isoform 1 of Splicing factor, arginine/serine-rich 7	0.89	1.12	0.90	0.81	0.82	0.72	0.80	8.6E-04	1.13
898	1.75	2	7.7	00480027	KIAA0649 Protein KIAA0649	1.15	1.05	0.73	0.85	0.70	0.80	0.77	3.8E-04	1.13
199	7.07	6	22.1	00218038	FAM10A4 Protein FAM10A4	0.91	1.19	0.89	0.82	0.75	0.67	0.76	6.5E-03	1.21
292	4.69	4	16.1	00790214	RCN2 Uncharacterized protein RCN2	0.93	0.87	0.73	0.70	0.86	0.80	0.76	8.6E-03	1.21
44	20.95	14	20.9	00011454	GANAB Isoform 2 of Neutral alpha-glucosidase AB precursor (glucosidase II)	0.98	0.94	0.74	0.74	0.80	0.80	0.75	2.3E-11	1.07
152	8.67	6	12	00000877	HYOU1 Hypoxia up-regulated protein 1 precursor	0.93	0.92	0.76	0.72	0.84	0.79	0.75	1.1E-08	1.09

^a Identification ranking based on the Prot score. Contaminants were excluded from the final list (see Materials and Methods).
^b Protein identification confidence calculated from peptides shared with no other protein (for a Prot score of >2, the identification confidence was >99%).
^c That is, the number of distinct peptides that allowed protein identification.
^d That is, the protein sequence covered by the peptides.
^e IPI, International Protein Index accession number.
^f Proteins indicated in boldface were included in the functional analysis.
^g The global *nef*-deleted/WT ratio from four experimental ratios per peptide (95% confidence interval within RATIO×EF and RATIO/EF) was determined. The statistical significance of the differences between the global ratio and 1, *t* test, bimodal, and *P* values is indicated. A dark gray background indicates a ≥1.2-fold increase; a light gray background indicates a ≥1.2-fold decrease.

down was significantly less infectious than WT virus produced in control cells. The 72% decrease in Nef potency calculated from these results (Table 2) confirms that Ezrin and EHD4 are key factors that regulate the ability of Nef to increase HIV-1 infectivity. Of note, simultaneous overexpression of Ezrin and EHD4 in virus-producing cells did not impact on Nef potency (data not shown).
In order to verify these data with viruses produced in T cells, WT and *nef*-deleted viruses were produced in SupT1 cell lines depleted of Ezrin and/or EHD4. Cell lines were generated with specific shRNA constructs and whole-cell lysates were analyzed by Western blotting in order to confirm targeted proteins silencing (Fig. 6A). Viral stocks produced from these cell lines and normalized for p24 content were assayed on HeLa-CD4 cells and Nef

potency was calculated as indicated in Materials and Methods. As shown in Fig. 6B, silencing of targeted proteins decreased Nef potency, but no additive effect could be concluded at that point. Although Ezrin silencing invariably decreased Nef potency, this effect was not considered significant due to variations of its magnitude between the three independent replicates. SupT1 cell lines were also infected with similar amounts of WT or *nef*-deleted replication competent viruses and viral spread was followed over 25 days. In this experiment, viruses were VSV-G pseudotyped to ensure similar entry efficiency in SupT1 cells. As expected, WT viruses replicated more efficiently than *nef*-deleted viruses in the parental cell line (Fig. 6C, upper left). EHD4 depletion affected the magnitude of WT and *nef*-deleted virus replication to the same extent and the gap between the percentages of infected cells was

TABLE 2 Host cell proteins involvement in the biogenesis of infectious HXBc2-pseudotyped HIV-1 particles and Nef potency^a

Protein targeted ^b	Mean $\pm$ SD ^c			
	Protein overexpression		Protein silencing	
	Nef-deleted virus infectivity	Nef potency	Nef-deleted virus infectivity	Nef potency
Mock	1.00	5.88 $\pm$ 1.35	1.00	6.60 $\pm$ 0.60 (100)
Gluc II long	1.12 $\pm$ 0.32	5.26 $\pm$ 0.61	ND	ND
Gluc II short	1.00 $\pm$ 0.15	5.16 $\pm$ 0.71	ND	ND
CD81	0.26 $\pm$ 0.14*	7.10 $\pm$ 2.78	2.03 $\pm$ 0.13*	6.38 $\pm$ 0.59 (96)
ALG-2	1.55 $\pm$ 0.14*	5.17 $\pm$ 0.79	0.87 $\pm$ 0.16	6.83 $\pm$ 0.49 (104)
Ezrin	1.10 $\pm$ 0.69	7.72 $\pm$ 4.15	1.98 $\pm$ 0.03*	5.39 $\pm$ 0.26* (78)
EHD4	0.78 $\pm$ 0.12*	6.23 $\pm$ 0.33	0.51 $\pm$ 0.04*	4.93 $\pm$ 0.39* (70)
Mock	ND	ND	1.00	5.22 $\pm$ 0.41 (100)
Ezrin/EHD4	ND	ND	1.05 $\pm$ 0.26	2.18 $\pm$ 0.21** (28)

^a Nef-deleted virus infectivity values were normalized to that of virus made in mock-transfected cells. WT virus infectivity normalized to that of nef-deleted viruses. Nef potency values were normalized to those of virions made from mock-siRNA-treated cells calculated as $(NP_{si} - 1)/(NP_{Mock} - 1) \times 100$, where NP_{si} and NP_{Mock} represent the Nef potency calculated in specific-siRNA- and mock-siRNA-treated cells, respectively. These values are indicated in parentheses in the last column.

^b Gluc II long and Gluc II short, the β chain of glucosidase II overexpressed together with either the short or long α chain, as indicated.

^c Data are presented as means calculated from at least three independent experiments. Asterisks indicate values significantly different from that obtained in mock conditions as determined by the Student *t* test: *, $P < 0.05$; **, $P < 0.005$. ND, not determined.

similar to that observed in the parental cell line (Fig. 6C, upper right). On the contrary, in SupT1 cells depleted of Ezrin, either alone or in combination with EHD4, the replication kinetics of WT and nef-deleted virus were identical in early time points (Fig. 6C, lower left and lower right, respectively), confirming the involvement of Ezrin in the replication advantage of WT viruses over nef-deleted viruses.

Finally, we analyzed the involvement of glucosidase II in HIV-1 infectivity. HXBc2 Env-pseudotyped virions were produced in the presence or absence of Nef from 293T cells transfected with constructs encoding the glucosidase II β chain and the long or the short α chain. Expression of glucosidase II was readily detectable in cell lysates and affected neither Gag processing in virion-producing cells nor virus infectivity (data not shown).

DISCUSSION

More than the presence of Nef itself in viral particles, it is thought that Nef leaves its imprint in virions in the course of their biogenesis, which accounts for the higher infectivity of WT virus over nef-deleted virus (39–41). Nef was shown to modulate the lipid composition of HIV-1 membrane; however, this could not explain the effect of Nef on virus infectivity (69). In the present study, we performed a proteomic analysis on WT and nef-deleted HIV-1 particles and observed that Nef regulates the incorporation of certain host cell-derived proteins into virions. Among these proteins, Ezrin and EHD4 appear to be connected to the ability of Nef to increase virus infectivity.

The use of iTRAQ allowed the identification of $\sim 1,000$ host cell proteins associated to viral particles, among which 105 were previously identified by others (42, 43, 46, 47), including (i) Tsg101, AIP-1/Alix, CHMP, and Vps proteins required for virus budding, (ii) clathrin, whose involvement in virus morphogenesis and infectivity has been described recently (70, 71), and (iii) transmembrane proteins acquired during the budding process such as

adhesion and MHC molecules. Besides the absolute number of host-derived proteins identified in virions, more differences were found between the protein composition of WT and nef-deleted viruses by iTRAQ than by DIGE. This was expected given the higher sensitivity of iTRAQ and is in agreement with results reported in a different context (72). Intriguingly, none of Nef ligands were enriched in WT virions, possibly due to the labile interaction with Nef. Alternatively, there might be two pools of Nef molecules in infected cells: (i) a functional pool directed to subcellular compartments where Nef interacts with cellular partners and (ii) an unproductive pool that fails to be translocated to such compartments and is passively incorporated into virions.

FIG 3 Manipulation of Ezrin or CD81 expression levels in virus-producing cells. HXBc2 Env or VSV-G pseudotyped GFP-reporter viruses were made in the presence or in the absence of Nef (Nef and Nef Xho, respectively) from cells transfected with control, CD81, or Ezrin-specific siRNA (A and B) or pCDNA3.1-, CD81-, or Ezrin-encoding plasmids (C and D). (A and C) Portions (15 μ g) of virus-producing cell lysate protein were resolved by SDS-PAGE, followed by Western blotting and immunodetection with the indicated antibodies. (B and D) HeLa-CD4 cells were incubated with the indicated viruses (~ 50 or 10 ng of p24 of HXBc2 Env or VSV-G-pseudotyped viruses, respectively), and virus infectivity was calculated by normalizing the percentage of GFP-positive cells to that measured when cells were incubated with viruses produced from cells transfected with control siRNA or plasmid in the absence of Nef. Representative results of experiments performed three times in duplicate are shown in panels A and C. Panels B and D present averages $\pm$ the standard deviations (SD) calculated from three independent experiments. Asterisks indicate a WT/nef-deleted virus infectivity ratio significantly different from that obtained in mock conditions (Student *t* test; *, $P < 0.05$).

FIG 4 Manipulation of EHD4 or ALG-2 expression levels in virus-producing cells. Virions were produced as in Fig. 3 from cells transfected with control or EHD4- or ALG-2-specific siRNA (A and B) or pCDNA3.1-, EHD4-, or ALG-2-encoding plasmids (C and D). (A and C) Cell lysates were analyzed as described in Fig. 3A and C with EHD4- and ALG-2-specific antibodies. (B and D) Virus infectivity was measured as in Fig. 3B and D. Unlike endogenous EHD4 detection, which required enhanced chemiluminescence (ECL) plus reagents (A, upper panel), overexpressed EHD4 was readily detected with ECL reagents; however, under these conditions, endogenous EHD4 appeared as a faint band (C, upper panel). Representative results of experiments performed three times in duplicate are shown in panels A and C. Panels B and D present averages $\pm$ the SD calculated from three independent experiments. Asterisks indicate a WT/*nef*-deleted virus infectivity ratio significantly different from that obtained in mock conditions (Student *t* test; *, $P < 0.05$).

The fact that no function has been attributed to virus-borne Nef molecules favors this latter hypothesis. Nevertheless, our work demonstrates that Nef shapes virions in the course of their biogenesis. Among specific differences between WT and *nef*-deleted virions, we found that glucosidase II was enriched in WT virions and yet did not affect virus infectivity (data not shown). CD81 and ALG-2 were significantly enriched in *nef*-deleted virions and affected virus infectivity independently of Nef. Finally, Ezrin and EHD4 were enriched in *nef*-deleted virions, and their expression levels in virus-producing cells affected virus infectivity and Nef potency, suggesting their contribution to the mechanisms that render WT viruses more infectious than *nef*-deleted viruses.

Glucosidase inhibitors negatively affect HIV-1, dengue virus, and hepatitis C virus infectivity (73–75). Here, we have demon-

FIG 5 Simultaneous extinction of Ezrin and EHD4 in virus-producing cells. (A) HXBc2 Env-pseudotyped GFP reporter viruses produced in 293T cells were purified as in Fig. 1, and pelleted material was analyzed by SDS-PAGE, followed by immunodetection to visualize p24, Ezrin, EHD4, and HA-tagged Nef. (B to D) Virions were produced from 293T cells transfected with control or EHD4 and Ezrin siRNA. Cell lysates and pelleted virions were analyzed by Western blotting, followed by immunodetection with EHD4, Ezrin, and p24-specific antibodies as indicated (B). The virus concentration was measured in the supernatants of transfected cells and normalized to that of virions produced from control siRNA-treated cells in the absence of Nef (D). HeLa-CD4 cells were incubated with increasing amounts of each virus, and the percentage of GFP-positive cells was measured by fluorescence-activated cell sorting (FACS) at 60 h postinfection (D, left panels). Virus infectivity was normalized to that of virus produced from cells transfected with control siRNA in the absence of Nef as in Fig. 3B (D, right panel). Representative results of experiments performed twice (A) or three times (B and D, left panel) are shown. Panels C and D present the averages $\pm$ the SD calculated from three independent experiments. Asterisks indicate a WT/*nef*-deleted virus infectivity ratio significantly different from that obtained in mock conditions (Student *t* test; **, $P < 0.005$).

strated glucosidase II enrichment in WT virions by two independent methods, suggesting a link with Nef potency; however, glucosidase II overexpression in virion-producing cells did not rescue the infectivity of *nef*-deleted virus (data not shown). Thus, its enrichment in WT virions probably does not account for the higher infectivity of WT viruses and rather suggests that Nef might target virus assembly to a glucosidase II-rich compartment, where this enzyme would be passively acquired. The other candidate proteins on which our functional characterization was focused were en-

FIG 6 Contribution of Ezrin and EHD4 to WT and *nef*-deleted HIV-1 biogenesis in SupT1 cells. (A) SupT1 cells transduced with vectors expressing Ezrin or EHD4-specific shRNAs, either separately or simultaneously were solubilized and $\sim 15 \mu\text{g}$ of cells lysate proteins were resolved by SDS-PAGE, followed by Western blotting and immunodetection with the indicated antibodies. The parental SupT1 cell line was analyzed in similar conditions. (B) WT and *nef*-deleted viruses encoding GFP were produced from the SupT1 cell lines described in panel A and incubated with HeLa cells. Virus infectivity was measured and Nef potency was calculated and normalized to that of virus produced from parental SupT1 cells as described in Table 2. (C) SupT1 cell lines were infected with replication-competent WT or *nef*-deleted viruses encoding GFP. At the indicated time points, cells were analyzed by FACS, and the percentages of GFP-positive cells were plotted as a function of time. Representative results of experiments performed three times are shown in panel C. Panel B presents the averages $\pm$ the SD calculated from three independent experiments. Asterisks indicate values significantly different from that obtained in mock conditions (Student *t* test; *, $P < 0.05$).

riched in *nef*-deleted virions. We found that HXBc2 Env-pseudotyped virus infectivity was negatively correlated to CD81 expression levels in virus-producing cells. The ~ 2 -fold increase of *nef*-deleted virus infectivity upon CD81 silencing (2.03 ± 0.13) was comparable to that reported by others (60). A similar correlation was observed in syncytia formation assays (76), suggesting that CD81 inhibits the fusion process mediated by HIV-1 Env and receptors. Of note, Nef potency was unchanged whether CD81 was overexpressed or silenced, which is consistent with the post-fusion effect of Nef (29). Thus, while the relative exclusion of CD81 renders virions more infectious, it is independent from the ability of Nef to increase virus infectivity. Based on the CD81-

Ezrin interaction (77, 78), the differential incorporation of CD81 into virions might be a consequence of the exclusion of Ezrin from WT virions (see below). ALG-2 was also suspected to restrict virus infectivity, based on its relative enrichment in the poorly infectious *nef*-deleted virions. Surprisingly, neither silencing nor overexpression of ALG-2 in virus-producing cells impacted on Nef potency, suggesting that ALG-2 enrichment in *nef*-deleted viruses is not what causes these viruses to be less infectious than WT viruses. As was concluded for CD81, the differential incorporation of ALG-2 into WT and *nef*-deleted virions might reveal the imprint of Nef on WT virion assembly. Nef has been shown to interfere with the biogenesis of the multivesicular body (79, 80). In this respect, Nef might affect the interaction between AIP-1/Alix and its ligands such as ALG-2. This could explain the relative exclusion of ALG-2 from WT virions, despite equivalent amounts of AIP-1/Alix incorporated into WT and *nef*-deleted virions.

The proteomic analysis revealed that EHD4 and Ezrin are relatively enriched in *nef*-deleted virions. Ezrin is a protein that switches between a soluble and membrane-bound conformation where it is involved in shaping the plasma membrane by connecting transmembrane cargos to the actin cytoskeleton (for a review, see reference 81). EHD4 is found in inner structures of the cell and regulates the traffic of endocytic vesicles (82). The role of ERMs in HIV-1 infection has been investigated mostly in target cells. Although some reports have demonstrated that Ezrin restricts HIV-1 infection (63), others have shown that ERMs are required to promote efficient HIV-1 entry (83). Here we found that Ezrin silencing in virus-producing cells increased progeny virus infectivity. Moreover, this effect was more pronounced on *nef*-deleted than on WT viruses, which resulted in a 22% decrease of Nef potency. The fact that Ezrin depletion decreased Nef dependence suggests that virus borne Ezrin is an obstacle to the completion of early steps in HIV-1 replication and that its exclusion from virions in the presence of Nef accounts, at least partially, for the ability of Nef to increase HIV-1 infectivity. Given that ERMs multimerize (for a review, see reference 81), target cell Ezrin might interact with virus-borne Ezrin molecules and interfere with the completion of postfusion steps, which is in agreement with the postfusion restriction witnessed on *nef*-deleted virions. Unlike Ezrin, EHD4 could not be considered as a virus-borne inhibitor. Indeed, siRNA treatment which depleted progeny virions from EHD4 decreased both virus infectivity and Nef potency. This likely reflects the involvement of EHD4 in the biogenesis of infectious virions and in the cellular mechanisms usurped by Nef to increase virus infectivity. As discussed below, EHD4 silencing might impact on the trafficking of a cellular protein involved in Nef potency.

In agreement with experiments conducted in 293T cells, Ezrin and EHD4 silencing in SupT1 cells lines used to make WT and *nef*-deleted virions decreased Nef potency by up to $\sim 30\%$. Although this effect was less pronounced than in 293T cells, it confirms the role of Ezrin and EHD4 in the Nef-phenotype. Ezrin and EHD4 silencing also affected the replication advantage of WT viruses over *nef*-deleted virus in SupT1 cells, especially at early time points of the replication kinetics. Interestingly, although EHD4 silencing decreased Nef potency in single-round infection, it did not affect the replication advantage of WT viruses over *nef*-deleted viruses, confirming that the growth advantage of WT viruses is not solely dependent on the ability of Nef to increase virus infectivity.

Also, there was a striking contrast between the efficiency of Ezrin and EHD4 silencing and the resulting partial effect on Nef

potency. It is thus possible that other cellular factors take part in the complex mechanism that renders WT viruses more infectious than their *nef*-deleted counterparts. In a recent work, the expression of MLV glycogag in virus-producing cells and its incorporation into HIV-1 particles was shown to substitute for the absence of Nef and has suggested "... convergent evolution in which two structurally unrelated proteins provide a function necessary for virion infectivity in lymphoid cells" (58). Another hypothesis is that glycogag has properties similar to the cellular factor that Nef accumulates in HIV-1 particles, potentially responsible for the net increase of virus infectivity. Based on our results, such a factor could be rerouted to virions through a EHD4 and/or Ezrin-dependent pathway hijacked by Nef. Given the involvement of EHD4 in the recycling compartment of the endocytic network (50), a plasma membrane-resident protein could be the target of Nef.

Interestingly, Ezrin overexpression did not alter the ability of Nef to increase virus infectivity. In addition to its effect as a virus-borne inhibitor, Ezrin might inhibit the functions of Nef in the course of virus biogenesis through its involvement in a multiprotein complex. Therefore, the use of dominant-negative forms of Ezrin might be another strategy to investigate its role in Nef potency. To this aim, virions were produced in the presence of plasmids encoding either full-length Ezrin or its FERM domain (amino acids 1 to 296), which acts as a dominant-negative form (for a review, see reference 81). Unlike full-length Ezrin, FERM expression decreased HIV release yet affected neither WT nor *nef*-deleted virus infectivity (data not shown), suggesting that the role of Ezrin on virus release and infectivity are genetically distinct. This approach might help identify dominant-negative forms of Ezrin capable of interfering with Nef potency. Similar conclusions could be drawn for EHD4 which overexpression did not affect Nef potency. Altogether, while Ezrin behaves as an inhibitor counteracted by Nef, EHD4 likely represents a Nef cofactor. This explains why cosilencing of both proteins in virus-producing cells has a compensatory effect on *nef*-deleted virus infectivity and an additive effect on Nef potency in 293T cells. The lack of additive effect in SupT1 cells might reflect the involvement of Ezrin and EHD4 in distinct steps of a common pathway.

Finally, there is a interesting phenotypic parallel between the impacts of *nef* deletion and the S₁₄₉A/S₁₇₈A mutations introduced into HIV-1 capsid described by Brun et al. (84). Indeed, both Nef and CA mutants present a defect in reverse transcription and infectivity that can be rescued by VSV-G pseudotyping. Nef-induced HIV-1 matrix phosphorylation was proposed to modulate virus infectivity (85); however, the involvement of matrix in Nef potency was later ruled out (86). Given that S₁₄₉ and S₁₇₈ in CA are phosphorylation sites (87), it is tempting to place Nef upstream of HIV-1 capsid Serine phosphorylation in the mechanisms that increase virus infectivity. The analysis of posttranslational modifications of viral proteins induced by Nef thus represents another starting point for further investigation.

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no. 2949) from Ronald Swanstrom, and HIV-1_{SF2} p24 antiserum (catalog no. 4251).

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