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 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 cytopathic 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-1JR-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-
fests 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, pBR431eG-nef-+, and pBR431eG-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 Sadowî (Institut des Neurosciences, Grenoble, France), respectively. The pSHR nef/LAI WT and the Nef XhoL are described elsewhere (39).

Small interfering RNA (siRNA) used in the present study (Ezrin, GCCG GAGAGUGCUAGAUUGUU; CD81, GCACCAAGUGCUAGAUUGUU; XhoL, GACAG GUGUGGACAUAGAUUU; and ALG-2, GACGAGGUGGACAUAGAUUU) 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 GeneTec (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 1:1,000 and 1:10,000, 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/75, 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 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% [w/vol] CHAPS [3-[3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate] 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 fluorescence 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 density of ~2 × 10⁶ cells/well and transfected after 48 h with 2 µg of a DNA mix containing the HIV-1 packaging plasmid (pCMVΔP1enVepA), the GFP-encoding HIV-1 vector (pHve2GFp), the HIV-1gag1XhoL Envelope glycoprotein-encoding plasmids (p5V1Henv), and either pSHR nef/LAI or the Nef XhoL construct at a 3:3:1:1 ratio, respectively. PHCMV-G and a Rev-encoding plasmid (1:1 mix) were substituted for p5V1Henv to generate VSV-G-pseudotyped virions. The medium was changed 24 h posttransfection, and supernatants were harvested after an additional 24-h
incubation time, centrifuged at 270 × 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 pBHR3.EGnef or pBR43 Ec-nef− (53, 54), respectively, and 1 µg of pHCMT1.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 mitomycin C 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 (∼4 × 10⁶ 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 × 10⁶ cells/ml. The cells were diluted 1:2 every 3 to 4 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 Thermofischer Scientific (Abgene, Epsom, United Kingdom). The mature shRNA sense sequences were as follows: Ezrin, CGTGCGATGCCTCAAAA GATAAT, and EHD4, CAGTGGCCTTTGGAAACGCTTT. 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 pHvec2.GFP plasmid with the appropriate plKO.1 plasmid. SupT1 cells were transfected with lentiviral vectors, either separately or in combination, and selected 72 h later in appropriate pLKO.1 plasmid. SupT1 cells were transduced with lentiviral as described above by substituting the pHIvec2.GFP plasmid with the dotyped shRNA viruses specific for Ezrin and EHD4 were made in 293T cells.

**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...
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/Alix 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 (NefXho) 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 (Table 2).

Ezrin and 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).
adverse effect was detected on Gag precursor synthesis or cleavage (Fig. 4A, lower panel). Interestingly, while ALG-2 silencing did not affect virus infectivity (Fig. 4B), EHD4 depletion specifically interfered with HXBc2 Env-pseudotyped virus biogenesis at two levels. siRNA induced a 2-fold decrease of nef-deleted virus infectivity (Fig. 4B) and decreased Nef potency by ~30% (Table 2), suggesting a link between EHD4 and the cellular mechanisms usurped by Nef to increase virus infectivity. EHD4 and ALG-2 were then overexpressed in virion-producing cells (Fig. 4C) and resulted in no increase or a ~2-fold increase of HXBc2 Env-pseudotyped nef-deleted progeny virus infectivity, respectively (Fig. 4D), whereas Nef potency remained unchanged (Table 2).

Impact of simultaneous silencing of Ezrin and EHD4 on virus infectivity and replication kinetics. In agreement with the proteomic analysis, Fig. 5A shows that Ezrin and EHD4 are less abundant in WT than nef-deleted viral pellets. We then sought to investigate the impact of simultaneous silencing of both proteins in virus-producing cells. For this purpose, WT and nef-deleted viruses were produced in 293T cell cotransfected with Ezrin and EHD4-specific siRNAs. Cell lysates and virus-associated proteins were analyzed by Western blotting, followed by immunodetection, which revealed efficient depletion of targeted proteins in both samples (Fig. 5B). Of note, protein silencing affected neither the p24 antibody-reactive pattern in virus-producing cells nor the amount of virus released in the cell culture supernatant (Fig. 5B and C). As shown in Fig. 5D, Ezrin and EHD4 silencing had a minimum impact on nef-deleted virus infectivity. On the contrary, WT virus produced from Ezrin and EHD4 double knock-
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

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**TABLE 1**

<table>
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<th>No.</th>
<th>Nef-Deleted WT ratio²</th>
<th>Control ratio</th>
<th>Experimental ratio</th>
<th>Nef-deleted WT ratio²</th>
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<td>1.06</td>
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<td>506</td>
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<td>0.04</td>
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</table>

² Identification ranking based on the Prot score. Contaminants were excluded from the final list (see Materials and Methods).

³ Protein identification confidence calculated from peptides shared with no other protein (for a Prot score of ≥ 2, the identification confidence was > 99%).

⁴ That is, the number of distinct peptides that allowed protein identification.

⁵ That is, the protein sequence covered by the peptides.

⁶ Table 2.

⁷ 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.

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

<table>
<thead>
<tr>
<th>Protein targetedb</th>
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<th>Protein silencing</th>
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<td></td>
<td>Nef-deleted virus infectivity</td>
<td>Nef potency</td>
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<td>Nef potency</td>
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</tr>
<tr>
<td>Mock</td>
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<tr>
<td>Glu II long</td>
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<td>5.16 ± 0.71</td>
</tr>
<tr>
<td>CD81</td>
<td>0.26 ± 0.14*</td>
<td>7.10 ± 2.78</td>
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<tr>
<td>ALG-2</td>
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<tr>
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</table>

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 (NPsi/P1)/(NPMock − 1) × 100, where NPsi and NPMock 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 Glu II long and Glu II short, the β chain of glucosidase II overlapped 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; and **, P < 0.005. ND, not determined.

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 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 ~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.
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). 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 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 demonstrated 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 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.
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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 ~15 μg 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 producing cells. The parental SupT1 cell line was analyzed in similar conditions. (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 was plotted as a function of time. Representative results of experiments performed three times are shown in panel C. Panel B presents the averages ± the SD calculated from three independent experiments. Asterisks indicate values significantly different from that obtained in mock conditions (Student t test; *, P < 0.05).

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 ~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 glycopag 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 glycopag 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, Ezrin 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 Vpx, Nef overexpression decreased HIV release yet affected neither WT nor nef-deleted counterparts. In a recent work, the expression of Ezrin and EHD4 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 glycopag 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.

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is a binding partner for HIV-1 p6 and ELAV p9 functioning in virus budding. Cell 114:689–699.


