Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions

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HIV buds from lipid rafts and requires cholesterol for its egress from and entry into cells. Viral accessory protein Nef plays a major role in this process. In this study, it not only increased the biosynthesis of lipid rafts and viral particles with newly synthesized cholesterol, but also enriched them. Furthermore, via the consensus cholesterol recognition motif at its C terminus, Nef bound cholesterol. When this sequence was mutated, Nef became unable to transport newly synthesized cholesterol into lipid rafts and viral particles. Interestingly, although its levels in lipid rafts were not affected, this mutant Nef protein was poorly incorporated into viral particles, and viral infectivity decreased dramatically. Thus, Nef also transports newly synthesized cholesterol to the site of viral budding. As such, it provides essential building blocks for the formation of viruses that replicate optimally in the host.

The negative effector (Nef) protein from human and simian immunodeficiency viruses is a membrane-associated myristoylated protein that measures 27–35 kDa (1–3). It is critical for high levels of viremia and the progression to AIDS in infected humans (4) and monkeys (5). This phenotype has been correlated with increased viral infectivity *in vitro*, which provides a convenient assay to study its effects in cultured cells (6, 7). This infectivity enhancement can be dependent on or independent of CD4 that serves as the receptor for viral entry. In the former case, Nef decreases the expression of CD4 on the cell surface, thereby increasing the incorporation of viral envelope (Env) proteins into virions (8). In the latter case, Nef still increases viral infectivity significantly (9, 10). This enhancement cannot be complemented by the expression of Nef in target cells. Although no differences were identified in major structural components and morphology between wild-type and mutant virions that lack Nef, ΔNef viruses displayed less efficient reverse transcription in target cells. Because Nef is expressed abundantly at the earliest stages of the viral replicative cycle (11), Nef could affect viral morphogenesis and budding to increase the fitness of the virus and facilitate its entry into recipient cells.

Lipid rafts, also known as detergent-resistant membranes (DRMs), are microdomains in the plasma membrane that are enriched in sphingolipids, cholesterol, and a subset of cellular proteins (12, 13). Two major pathways contribute to cholesterol homeostasis in mammalian cells (14). Most exogenous cholesterol, which originates from low-density lipoproteins, is internalized via coated pits and distributed to intracellular pools. In addition, cells can synthesize cholesterol in their endoplasmic reticulum when the uptake of exogenous cholesterol is blocked. The newly synthesized cholesterol is then transported into the Golgi apparatus and distributed to various intracellular pools. Because cellular cholesterol is compartmentalized, some sites (including DRMs) are enriched in this newly synthesized lipid (15, 16).

Previous studies determined that cholesterol is essential for the egress from and entry of HIV into cells (17–23). Indeed, Nef not only colocalized with viral structural components in the DRMs (24, 25), but its ability to increase viral infectivity depended on cholesterol (25). However, it remained undetermined which cholesterol pools played a major role in this process and if this lipid was necessary for viral replication.

To answer these questions, we studied effects of Nef on the metabolism of intracellular cholesterol as well as its delivery into lipid rafts and progeny virions. We found that Nef increases not only the transcription of cholesterogenic enzymes but also the biosynthesis of cholesterol. Nef bound this newly synthesized cholesterol and increased the levels of cholesterol in lipid rafts and progeny virions. The high content of cholesterol made the wild-type virions more infectious than their *nef*-defective counterparts.

Materials and Methods

Plasmids. The HIV proviral clone pH (11,515 bp) lacks 5′- and 3′-flanking cellular sequences in pNL4-3 (14,874 bp). In pH SF2Nef, NL Nef was replaced with SF2 Nef. CD8-SF2Nef fusion and CD8T (26) proteins were expressed from pEF-BOS. Nef was also expressed from pcDNA3 (Invitrogen). To express Nef in *Escherichia coli*, the Nef gene was cloned into pET-15b plasmid using its *Nde*I and *Bam*HI site (Novagen). This vector contains a 6xHis tag at the N terminus. QuikChange XL site-directed mutagenesis kits (Stratagene) were used to create various mutant *nef* constructions.

Viruses. HIV particles were produced from 293T cells (27). Viruses were quantitated by an ELISA for $p24^{Gag}$ content. To determine viral infectivity, GHOST-CXCR4/HIV-GFP indicator cells, which were stably transfected with the HIV-2 LTR driving GFP expression construct, were infected with the equal amounts of virus. Infection was quantified by determining the GFP fluorescence intensity by flow cytometry.

Isolation of DRMs. DRMs were isolated, and Western blotting and cholesterol determinations were performed as described (25, 28). Briefly, after being incubated with 1 ml of lysis buffer (0.5% Triton X-100/1 mM EDTA/1 mM Na_3VO_4/1 mM PMSF/10 μg/ml aprotinin/150 mM NaCl/25 mM Mes, pH 6.5) for 30 min at 4°C, cells were homogenized, diluted with 1.5 ml of 85% sucrose, and loaded to the bottom of an ultracentrifugation tube. Samples were overlaid with 5 ml of 35% sucrose and 4.5 ml of 5% sucrose and spun 24 h at 200,000 × *g* at 4°C in a Beckman SW-41 rotor. DRMs were visible at the 35%/5% sucrose interface and collected. The protein concentration of these fractions was determined by BCA protein assay kit (Pierce).

Determination of Cholesterol. Newly synthesized cellular cholesterol was determined by labeling cells with [^{3}H]mevalonic acid. Briefly, cells were labeled with [^{3}H]mevalonic acid [15 μCi/ml medium (1 Ci = 37 GBq)] in the medium containing 10% lipoprotein-poor serum. Cells were then lysed, and lipids were extracted into chloroform (25). Extracted lipids were separated on precoated TLC plates (Merck) in a solvent system containing chloroform, methanol, and acetic acid at 95:4:1 ratio. [^{3}H]Cholesterol (Amersham Biosciences) was used as the reference sterol. Before exposure to

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Abbreviations: Nef, negative effector; DRM, detergent-resistant membrane; CRM, cholesterol recognition motif; CHO, Chinese hamster ovary.

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Hyperfilm (Amersham Biosciences), TLC plates were sprayed with Amplify Fluorographic Reagent (Amersham Biosciences). The radioactivity was also quantified by scintillation counting. To determine the content of newly synthesized cholesterol in DRMs and viral particles, DRMs or HIV viruses were isolated from cells labeled with [^{3}H]mevalonic acid and quantified by either total protein or viral p24^{Gag} level. Cholesterol was extracted and determined as above. The contamination by microvesicles, as determined from supernatants of cells expressing only Nef, was minimal (<10%).

***In Vitro* Cholesterol Binding.** Recombinant Nef proteins were produced and purified by the His bind purification kit (Novagen). Proteins were used directly for binding studies without elution. Beads were mixed with [17α-*methyl*-^{3}H]promegestone (1.2×10^4 nM, 84 mCi/mol, Perkin–Elmer) at a final concentration of 120 nM in 100 μl of PBS, in the absence or presence of serial increased level of cold water-soluble cholesterol (Sigma). The final concentration of the cold cholesterol was 0, 25, 250, or 2,500 μM, respectively. After incubation at 4°C for 30 min, samples were photoirradiated for 30 min with a UV cross-linker (Stratagene). Irradiated beads were then washed extensively with PBS and resuspended in SDS sample buffer. After boiling for 3 min, the samples were resolved by SDS/PAGE. The gel was first fixed in solution of isopropanol: acetic acid:water (25:10:65) for 30 min, treated with fluorographic Amplifier solution (Amersham Biosciences) for another 30 min, dried, and exposed to Hyperfilm (Amersham Biosciences).

***In Vivo* Cholesterol Binding.** The interaction between Nef and cholesterol in cells was determined as before (29). Briefly, 293T cells expressed CD8.Nef fusion proteins and were labeled with [17α-*methyl*-^{3}H]promegestone at 4,000 cpm/ml for 48 h in medium containing 2% FBS. After photoirradiation for 30 min, cells were lysed. A mouse anti-human CD8 monoclonal antibody (32-M4) (Santa Cruz Biotechnology) was conjugated to Dynabeads (Dynal, Great Neck, NY) according to the manufacturer's protocol and used to immunoprecipitate CD8 fusion proteins from cell lysates. After extensive washing, the radioactivity was directly determined from the beads by the scintillation counter.

Immunoblotting. The mouse anti-p24^{Gag} (183-H12–5C) monoclonal antibody and rabbit anti-Nef polyclonal antibody were from the National Institutes of Health AIDS Research and Reference Reagent Program. The mouse anti-Nef monoclonal antibody was a kind gift of Casey Morrow (University of Alabama, Birmingham). The rabbit anti-CD8 polyclonal antibody (H-160) was from Santa Cruz Biotechnology. These antibodies were used as the first antibodies for immunoblotting. GM1 was detected by staining with horseradish peroxidase-conjugated cholera toxin B (Sigma). Detection of horseradish peroxidase-conjugated antibodies and cholera toxin B was performed using enhanced chemiluminescence (Amersham Bioscience).

Results

Nef Increases the Synthesis of Cholesterol in Cells. To study how Nef affects cholesterol homeostasis in cells, three Chinese hamster ovary (CHO) cell lines were selected. In wild-type K1 cells, all pathways operate. Mutant Mef18B-2 cells do not express the low density lipoprotein receptor and cannot take up exogenous cholesterol (30). Mutant 215 cells do not express the biosynthetic 4-carboxysterol decarboxylase and cannot synthesize cholesterol (31). All three cell lines were cultured in medium containing 10% normal FBS under normal culture conditions, and Nef or the empty plasmid vector was expressed transiently. Subsequently, total cholesterol was extracted and quantified. As presented in Fig. 1*a*, Nef increased significantly levels of total cholesterol in K1 and Mef18B-2 but not in 215 cells (compare lanes 1 and 3 to lane 5). Of note, levels of total cellular cholesterol are highly regulated and cannot vary by multiples or other orders of magnitude (14). Thus,

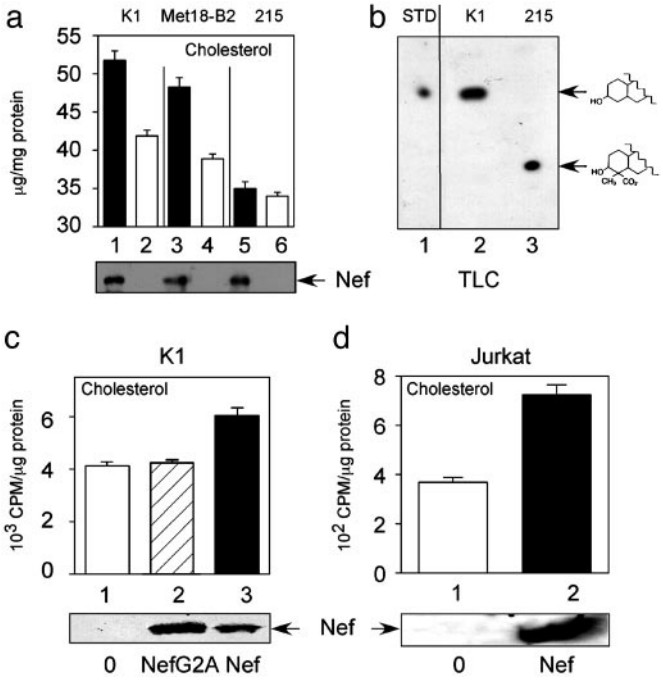

Fig. 1. Nef increases the synthesis of cholesterol in cells. (*a*) Nef does not affect the uptake of exogenous cholesterol. Three CHO cell lines, K1, MET18B-2, and 215 cells, were transfected with pcDNA (white bars) or pcDNA-Nef (black bars). Total cellular cholesterol was quantified by an enzyme-linked assay. Levels of cholesterol were normalized to amounts of total cellular protein. (*b*) Detection of newly synthesized cholesterol. CHO cells were labeled with radioactive cholesterol precursor, [^{3}H]mevalonic acid. Newly synthesized labeled cholesterol was visualized by autoradiography following TLC. The top arrow denotes cholesterol and the lower arrow denotes 4-carboxysterol. (*c*) Nef increases the synthesis of cholesterol in CHO cells. CHO K1 cells, which expressed the empty plasmid vector (0, white bar), wild-type (black bar), or mutant myristoylation-negative NefG2A (striped bar) proteins were labeled with [^{3}H]mevalonic acid. Newly synthesized labeled cholesterol was quantified using a scintillation counter and normalized to levels of total cellular protein. (*d*) Nef increases the synthesis of cholesterol in Jurkat cells. Newly synthesized cholesterol from Nef-inducible Jurkat cells, which were labeled with [^{3}H]mevalonic acid, was quantified as above. In all experiments, the expression of Nef was confirmed by Western blotting, which is denoted by arrows in *a*, *c*, and *d*, *Lower*. Standard deviations from the mean reflect three independent experiments.

because Nef increased levels of cellular cholesterol only when the biosynthetic machinery was intact, this finding suggested that it affects the biosynthesis of cholesterol and/or its transport.

To study this endogenous pathway in more detail, cells were cultured in medium containing 10% lipoprotein-poor serum and labeled with the radioactive cholesterol precursor [^{3}H]mevalonic acid (31). Total lipids were extracted, and the newly synthesized cholesterol was visualized by TLC. As presented in Fig. 1*b*, lane 2, wild-type K1 cells synthesized cholesterol from [^{3}H]mevalonic acid. However, in mutant 215 cells, the predominant product was the cholesterol intermediate 4-carboxysterol (cholesten-4β-methyl,4α-carboxy,3β-ol) (Fig. 1*b*, lane 3). Of note, all newly synthesized cholesterol was in the unesterified form (Fig. 1*b*, lanes 2 and 3). Similar results were observed in 293T and Jurkat cells (data not presented). Thus, the radioactivity in total lipids correlates directly to the level of newly synthesized cholesterol in wild-type cells.

When K1 cells expressed Nef or a mutant Nef protein lacking the N-terminal myristoylation signal (NefG2A), only the wild-type protein increased levels of endogenous cholesterol (Fig. 1*c*, lane 3). The mutant NefG2A protein had no effect (Fig. 1*c*, lane 2). Similar experiments were also performed in Jurkat cells, where Nef was not only integrated stably but whose expression could be induced with

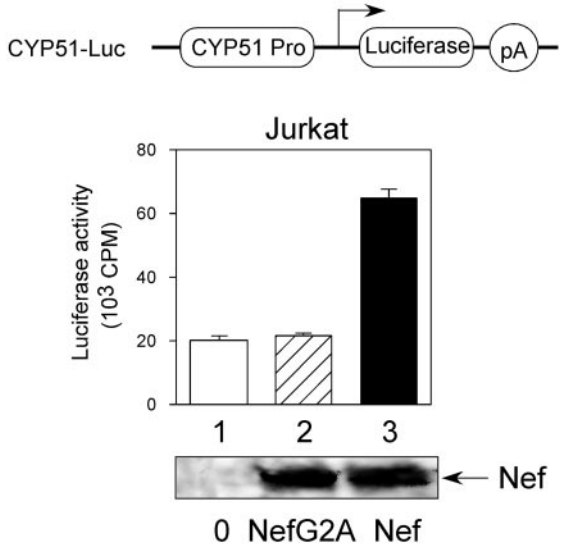

Fig. 2. Nef increases the expression of the CYP51 gene in Jurkat cells. (*Top*) The CYP51 promoter directed the expression of the luciferase reporter gene and is presented schematically. (*Middle*) Luciferase activity was determined in cell lysates of Jurkat cells. (*Bottom*) The expression of Nef proteins, denoted by an arrow, was confirmed by Western blotting. Standard deviations from the mean are representative of three independent experiments.

tetracycline (24). It was important to perform these experiments in human T cells, which are the natural host for HIV. Indeed, Nef increased the synthesis of cholesterol in Jurkat cells. As presented in Fig. 1*d*, 2-fold increased radioactivity was observed in induced versus uninduced Jurkat cells (compare lanes 1 and 2).

Nef Increases the Expression of the CYP51 Gene in Jurkat Cells. To determine how Nef perturbs this cholesterogenic pathway, we studied the expression of the cytochrome P450 51 (CYP51) gene, whose transcripts were increased by Nef in cDNA microarrays in Jurkat cells (32). It is known that Nef activates T cell signaling cascades and causes cytoskeletal rearrangements (26, 33, 34). CYP51 encodes the sterol 14α-demethylase gene, which catalyzes the demethylation of lanosterol during cholesterol biosynthesis (35). The promoter of CYP51 was placed upstream of the luciferase reporter gene (Fig. 2 *Top*). When Nef and the CYP51-luciferase were coexpressed in Jurkat cells, we observed 3-fold increased luciferase activity over that with the mutant NefG2A protein and the empty plasmid vector (Fig. 2, compare lane 3 to lanes 1 and 2). Thus, Nef augments the expression of at least one cholesterogenic gene to increase the synthesis of cholesterol.

Nef Increases Levels of Newly Synthesized Cholesterol in Lipid Rafts and HIV Particles. To determine if newly synthesized cholesterol is incorporated into lipid rafts and progeny virions and if Nef affects the intracellular trafficking of cholesterol, we studied different HIV proviruses in cells that support abundant expression of viral genes. First, three different proviruses, one wild type, the other lacking the *nef* gene, and the third encoding the mutant NefG2A protein, were placed into 293T cells. Cells were then labeled with [^{3}H]mevalonic acid, and lipid rafts were isolated 2 days later.

The purity of the lipid raft fractions was first confirmed by a control experiment showing the localization of CD45. CD45 is a transmembrane tyrosine phosphatase constitutively expressed on all nucleated hematopoietic cells. It is excluded from lipid rafts (36). When CD45-expressing cells were fractionated, CD45 was detected only in the cytosol and plasma membrane and not in DRMs (Fig. 3*a Lower*, compare lanes 1–3), although ganglioside M1 (GM1), a general marker for lipid rafts, was readily detected (Fig. 3*a Upper*, lane 1). Second, Nef and its mutant counterpart both distributed

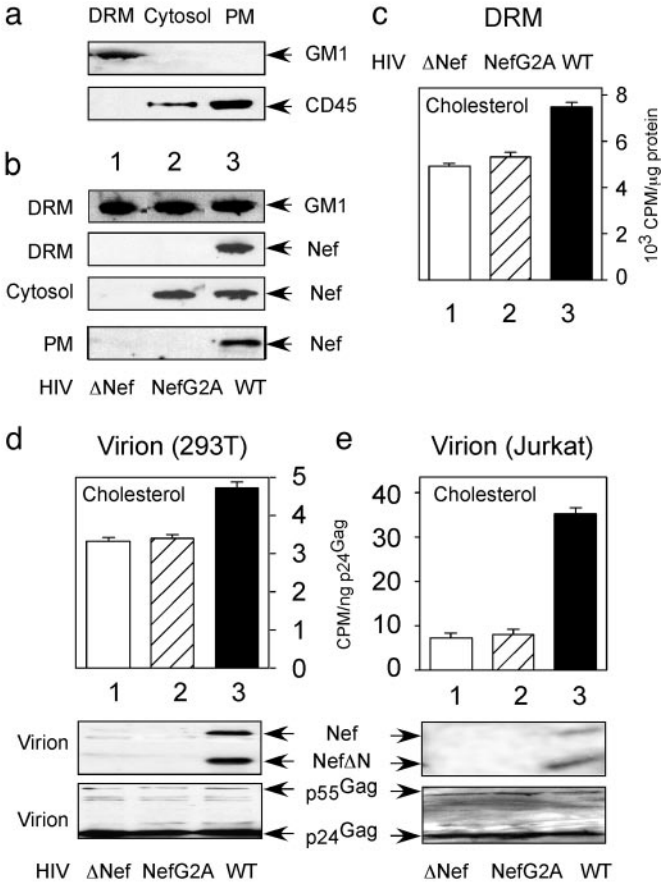

Fig. 3. Nef increases levels of newly synthesized cholesterol in lipid rafts and HIV particles. (*a*) Isolation of DRMs from cells. 293T cells were transfected with a CD45-expression plasmid, and three fractions were isolated: DRM, cytosol, and plasma membrane (PM). They were analyzed with the cholera toxin B, which detects GM1, or the anti-CD45 monoclonal antibody by Western blotting. (*b*) Distributions of the wild-type Nef and mutant NefG2A protein in different fractions. 293T cells were transfected with three HIV proviruses, the wild-type (HIV WT), and those lacking Nef (HIV ΔNef) or expressing Nef without the myristoylation site (HIV NefG2A), respectively. Cells were then fractionated, and different fractions were analyzed with the cholera toxin B or the anti-Nef monoclonal antibody by Western blotting. (*c*) Nef increases levels of newly synthesized cholesterol in lipid rafts. 293T cells were transfected with three proviruses as in *b* and labeled with [^{3}H]mevalonic acid. DRMs were isolated and quantified by scintillation counting. The radioactivity was normalized to levels of total cellular protein. Bars represent the following: white, HIV ΔNef; striped, HIV NefG2A; black, HIV Nef. (*d*) Nef increases levels of newly synthesized cholesterol in HIV particles from 293T cells. Viruses, collected from the 293T cells and labeled with [^{3}H]mevalonic acid, were purified, and labeled cholesterol isolated from these particles was quantified by scintillation counting. Radioactivity was normalized to levels of p24^{Gag} (*Top*). The expression of Nef and Gag proteins from these viruses was confirmed by Western blotting (*Middle* and *Bottom*). NefΔN was cut by the viral protease and lacks the N terminus of Nef. (*e*) Nef increases levels of newly synthesized cholesterol in HIV particles from Jurkat cells. As in *d*, levels of newly synthesized cholesterol, which were normalized to amounts of p24^{Gag}, are presented (*Top*). The expression of Nef and Gag in these viruses, confirmed by Western blotting, is presented (*Middle* and *Bottom*) and denoted by arrows. Standard deviations from the mean are from three independent experiments (*c*–*e*).

correctly in these fractions (Fig. 3*b*, lower three panels, lanes 2 and 3). Wild-type Nef protein was detected in all three fractions, which include the DRM, cytosol, and plasma membrane (Fig. 3*b*, lane 3). However, because it is unable to target membranes, the mutant NefG2A protein was only detected in the cytosol (Fig. 3*b*, lane 2).

When levels of newly synthesized cholesterol were measured in cells transfected with different proviruses, radioactivity was recov-

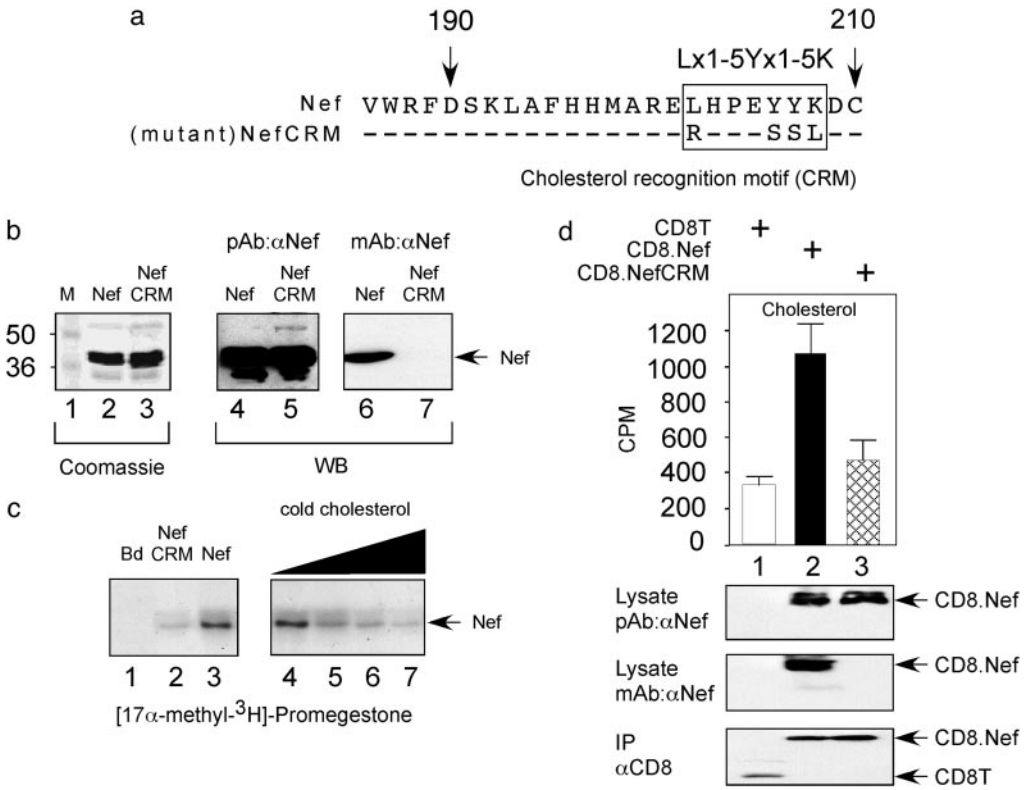

Fig. 4. Nef binds cholesterol *in vitro* and *in vivo*. (*a*) A putative CRM (37) in Nef. The upper sequence represents the C terminus of wild-type Nef from HIV-1_{SF2}. The lower sequence contains the mutations introduced to disrupt this motif. (*b*) Expression of recombinant Nef proteins from *E. coli*. Wild-type Nef and mutant NefCRM proteins were expressed in the pET system and purified by His Bind Resins. The purity of these proteins is demonstrated by SDS/PAGE followed by Coomassie blue staining (*Left*). Western blotting (WB), using polyclonal (pAb) and monoclonal (mAb) antibodies against Nef, is presented (*Center* and *Right*, respectively). Arrows denote different Nef proteins. (*c*) Nef binds cholesterol *in vitro*. Binding of cholesterol to purified Nef proteins was determined after incubating them with photoactivatable [17α-*methyl*-^{3}H]promegestone in the absence and presence of increased levels of cold cholesterol. Labeled bands were visualized by autoradiography after SDS/PAGE. Arrow denotes Nef with bound [^{3}H]promegestone (Bd, beads alone). (*d*) Nef binds cholesterol *in vivo*. 293T cells expressed truncated CD8T, which contains the extracellular and transmembrane domains of CD8 (white bar), wild-type CD8.Nef (CD8T fused to Nef, black bar), and mutant CD8.NefCRM (CD8T fused to NefCRM, cross-hatched bar) chimeras. Cells were then labeled with the photoactivatable [17α-*methyl*-^{3}H]promegestone and immunoprecipitated with the anti-CD8 antibody. The expression of these proteins was confirmed with anti-Nef and CD8 antibodies (*Lower*). Levels of [17α-*methyl*-^{3}H]promegestone on immunoprecipitated proteins were quantified with scintillation counting (*Upper*). Standard deviations from the mean are from three independent experiments (*d*).

ered in the DRM fraction in each case (Fig. 3*c*). However, counts were increased up to 40% only with the wild-type provirus (Fig. 3*c*, lane 3). Because HIV ΔNef expressed no Nef and the mutant NefG2A protein was restricted to the cytosol (Fig. 3*b*, lanes 1 and 2), these mutant proviruses did not increase levels of newly synthesized cholesterol in DRMs (Fig. 3*c*, lanes 1 and 2).

Next, we purified virions from supernatants and quantified the radioactivity in these particles after normalization for levels of the viral structural protein $p24^{Gag}$. The purity of these virions was determined by Western blotting using an anti-Gag antibody (Fig. 3*d*). Robust $p24^{Gag}$ and weaker $p55^{Gag}$ bands were detected (Fig. 3*d Lower*). The content of Nef proteins in these virions was also determined. Only the wild-type Nef protein was incorporated into virions. Note that Nef is cut by the viral protease in virions, and this N-terminally deleted Nef peptide migrates at a lower molecular weight (Fig. 3*d*, lane 3). Similar to results with lipid rafts, the newly synthesized cholesterol was incorporated into progeny virions, and this incorporation was increased up to 40% in the presence of Nef (Fig. 3*d*, compare lane 3 to lanes 1 and 2). This amount parallels the increase of newly synthesized cholesterol in DRMs.

Finally, we isolated these three virions from chronically infected Jurkat cells, which were labeled with [^{3}H]mevalonic acid. Up to 3-fold increased levels of newly synthesized cholesterol were found in viral particles in the presence of the wild-type Nef protein (Fig. 3*e*, compare lane 3 to lanes 1 and 2). We conclude that Nef increases not only the synthesis of but also the incorporation of newly synthesized cholesterol into lipid rafts and progeny virions in infected cells.

Nef Binds Cholesterol *in Vitro* and *in Vivo*. Because Nef increased the biosynthesis of cholesterol and was colocalized with viral structural proteins in lipid rafts, we wondered if Nef could also help transport this lipid into the DRM. Nef contains a C-terminal amino acid sequence, preserved in all clades of HIV (data not presented), which is similar to the cholesterol recognition motif (CRM) identified in several other cholesterol-binding proteins (37). To determine if Nef binds cholesterol *in vitro*, we expressed the wild-type Nef and mutant NefCRM proteins from *E. coli*. The mutant NefCRM protein contains four mutations, namely L202R, Y206S, Y207S, and K208L, which abolish this consensus CRM (Fig. 4*a*). Each recombinant protein was purified by His-affinity chromatography and examined by Coomassie blue staining after SDS/PAGE (Fig. 4*b*). With both proteins, a single band was detected at around 36 kDa (Fig. 4*b*, lanes 2 and 3), consistent with the size of Nef. A monoclonal anti-Nef antibody, which was directed against the C terminus of Nef, confirmed this mutation (Fig. 4*c*, compare lanes 5 and 7). To perform the binding study, we used the photoactivatable [17α-*methyl*-^{3}H]promegestone, which contains a side chain at C_{17} similar to that in cholesterol (37). When either Nef protein was incubated with [17α-*methyl*-^{3}H]promegestone and cross-linked by UV light, the radioactivity was detected easily on the wild-type Nef but not on the mutant NefCRM protein (Fig. 4*c*, compare lanes 2 and 3). Cold cholesterol competed for this binding (Fig. 4*c*, lanes 4–7), suggesting that the interaction between cholesterol and the CRM in Nef was also specific. Thus, Nef binds cholesterol *in vitro*.

Finally, we wanted to know whether Nef binds cholesterol *in vivo*. To perform this assay, we created new CD8.Nef fusion proteins. The expression of the wild-type CD8.Nef and mutant CD8.NefCRM chimeras was confirmed by Western blotting (Fig. 4*d*, lanes 2 and 3). 293T cells expressing the truncated CD8 protein (CD8T), wild-type CD8.Nef, and mutant CD8.NefCRM chimeras were labeled subsequently with [17α-*methyl*-^{3}H]promegestone. After UV irradiation, cells were lysed and immunoprecipitated with anti-CD8 antibodies, and the radioactivity was quantified. As

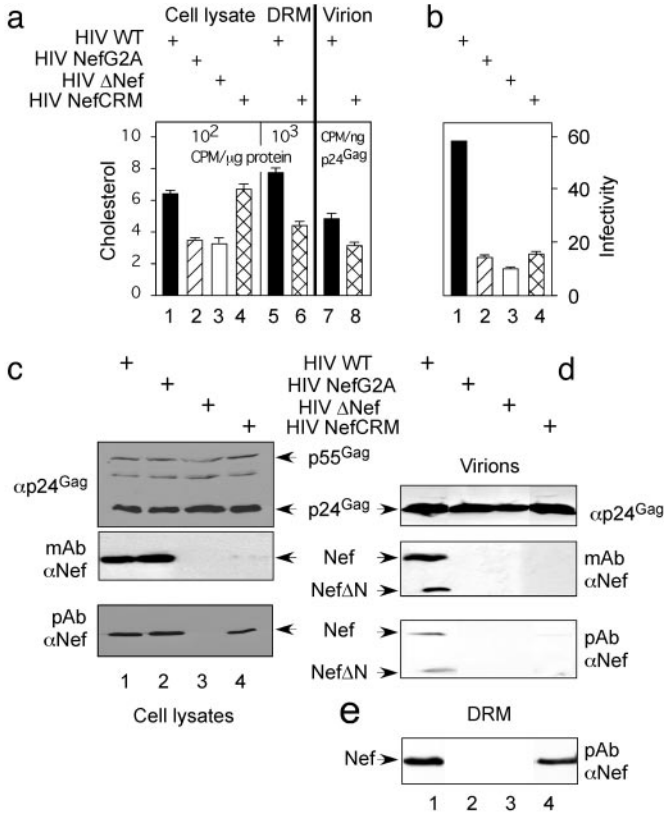

Fig. 5. CRM is required for Nef to increase HIV infectivity. (*a*) Increased levels of newly synthesized cholesterol in lipid rafts and viral particles by Nef depend on the CRM. 293T cells were transfected with four different proviruses expressing the wild-type Nef (HIV WT, black bar), mutant NefG2A protein (HIV NefG2A, striped bar), no Nef (HIVΔNef, white bar), or mutant NefCRM protein (HIV NefCRM, cross-hatched bar). Cells were labeled with [^{3}H]mevalonic acid. Labeled cholesterol from total cell lysates, DRM, and viral particles was extracted, quantified by scintillation counting, and normalized to levels of cellular protein or $p24^{Gag}$. Results are representative of three independent experiments. (*b*) Cholesterol binding to Nef increases HIV infectivity. GHOST indicator cells were infected with equal amounts of the same viruses as in *a*. Two days later, the expression of GFP in infected cells was quantified by flow cytometry. Results presented are representative of three independent experiments. (*c*–*e*) Expression levels of Gag and Nef in transfected cells (*c*) and virions (*d*) and of Nef in DRM were determined by Western blotting.

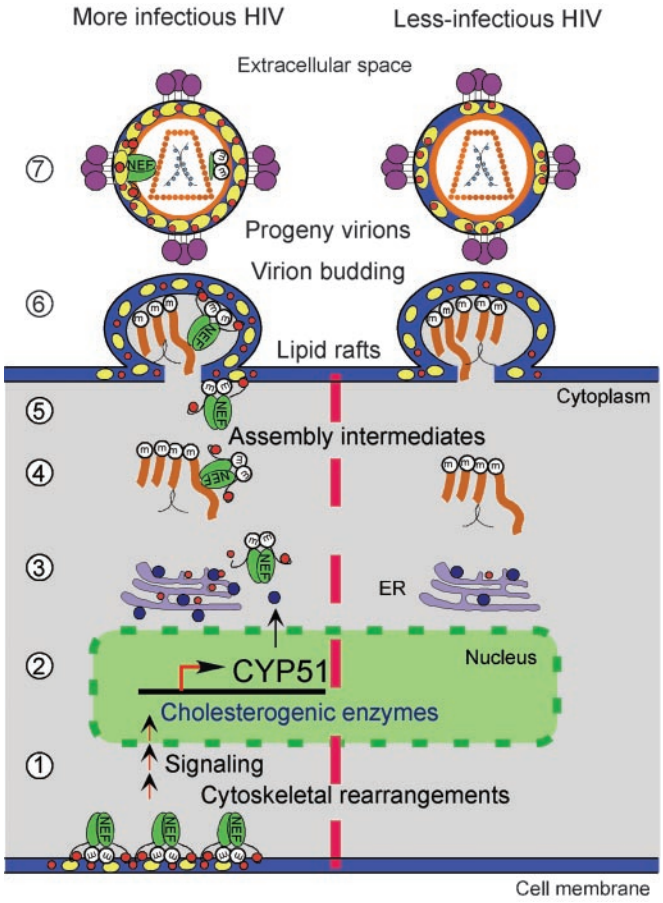

Fig. 6. A model for the effects of Nef on viral infectivity. Nef is expressed as an oligomer abundantly before viral structural proteins. It activates cellular signaling cascades and causes cytoskeletal rearrangements (step 1). These lead to increased transcription of at least one cholesterogenic enzyme, CYP51 (step 2). Increased CYP51 activity (blue circles) increases synthesis of cholesterol (red circles) in the endoplasmic reticulum (step 3). The model shows the transport of Nef in viral assembly intermediates (step 4) to lipid rafts (step 5). Nef and newly synthesized cholesterol are incorporated into DRM and virions (step 6). More infectious viral particles are released into extracellular space (step 7). Nef is also cleaved by the viral protease in virions (step 7).

presented in Fig. 4*d*, the CD8.Nef chimera exhibited 3-fold higher radioactivity than the control CD8T and the mutant hybrid CD8.NefCRM proteins (compare lanes 1–3). Thus, Nef binds cholesterol *in vitro* and *in vivo*.

Cholesterol Binding Is Required for Nef to Increase Viral Infectivity. Finally, to determine the importance of cholesterol binding to Nef for the viral replicative cycle, we examined effects of the mutant NefCRM protein on cholesterol biosynthesis. The same mutations in the *nef* gene were introduced into the provirus (HIV NefCRM). As presented in Fig. 5*a*, the mutant NefCRM protein retained the ability to increase the synthesis of cholesterol (compare lanes 1–4), but it did not increase levels of newly synthesized cholesterol in DRMs and viral particles (lanes 6 and 8). This result indicates that the transport of cholesterol by Nef also depends on its binding cholesterol *in vivo*. Second, we performed single-cycle infectivity assays. Four types of virions were produced from 293T cells: HIV WT, HIV NefG2A, HIV ΔNef, and HIV NefCRM. They produced similar levels of viral structural Gag proteins in transfected cells (Fig. 5*c Top*, lanes 1–4). Levels of Nef in HIV WT, HIV NefG2A, and HIV NefCRM were also comparable (Fig. 5*c Middle*, lanes 1, 2, and 4). However, only the polyclonal anti-Nef antibody recognized the mutant NefCRM protein in HIV NefCRM-infected cells (Fig. 5*c Middle* and *Bottom*, compare lanes 1, 2, and 4).

When equal amounts of these viruses were used to infect GHOST HIV LTR/GFP indicator cells, we observed significantly decreased numbers of GFP-positive cells among HIV NefCRM-infected cells (Fig. 5*b*, lane 4) compared with HIV WT-infected cells (Fig. 5*b*, lane 1). The florescence intensity of HIV NefCRM-infected cells was close to that of HIV NefG2A- and HIVΔNef-infected cells (Fig. 5*b*, lanes 2 and 3). Because virion incorporation is required for Nef to increase HIV infectivity, we further compared the levels of Nef in viral particles. We detected Nef only in wild-type particles (Fig. 5*d*). Similar to the mutant NefG2A protein, although its levels in the DRM were comparable to the wild-type Nef protein (Fig. 5*e*, lanes 1 and 4), the mutant NefCRM protein was incorporated poorly into virions (Fig. 5*d*, lowest panel, lane 4). We conclude that the disruption of the cholesterol binding significantly impairs the transport of cholesterol by Nef and the infectivity of HIV. These results confirm the important role of Nef as a cholesterol carrier in the viral replicative cycle.

Discussion

In this study, Nef increased cholesterol biosynthesis and its incorporation into DRMs and progeny virions. Importantly, Nef trans-

ported newly synthesized cholesterol to sites of viral budding and release. Concomitantly, viral infectivity was increased. These results suggest that Nef may increase viral infectivity by increasing the synthesis and incorporation of cholesterol into progeny virions and that newly synthesized cholesterol may participate in this process. A model incorporating these data is presented in Fig. 6.

The effects of Nef on cholesterol and viral infectivity were most pronounced in T cells, which are the natural host for HIV infection. Direct effects of Nef on the biosynthesis and incorporation of cholesterol were measured. Nef lacking the myristoylation signal and membrane targeting formed an important negative control. Nef bound cholesterol via its C terminus, which is conserved in all clades of HIV and whose mutation not only blocked the binding of cholesterol but decreased viral infectivity proportionally. Although it was limited to 2% of Nef, and the polyclonal anti-Nef antibody recognized the wild-type Nef as well as mutant NefCRM proteins equivalently, we cannot exclude other unrelated effects of our mutation. For example, the mutant NefCRM protein could have been misfolded, which would exclude it from virus particles. Alternatively, its N-terminal myristoylation and binding of cholesterol are both required for the tight association of Nef with progeny virions. Nevertheless, this study brings together several disparate observations on cholesterol homeostasis and viral infectivity and reveals that even in the presence of abundant exogenous cholesterol, the synthesis of new cholesterol can be increased in cells. Most likely, this pathway serves the needs of a rapidly replicating virus that must create many copies of itself before the cell dies.

Newly synthesized cholesterol is selectively transported to DRMs (15, 16). In the present study, newly synthesized cholesterol was incorporated more efficiently into virions than preformed cellular cholesterol. Moreover, we were unable to incorporate any exogeneous cholesterol into these structures (data not presented). This finding most likely reflects the association of cholesterol and Nef in transport complexes before reaching the cell surface. Because Nef also causes actin polymerization and cytoskeletal rearrangements (33), it could also help to form its own lipid rafts.

Another unexpected finding was the dependence of sterol binding on the C terminus of Nef. Mutation of the CRM abrogated the binding of Nef and cholesterol *in vitro* and *in vivo*. The consequent reduction of cholesterol in virions was associated with a significant decrease in viral infectivity. Similar cholesterol-binding sites have been identified in several membrane proteins, which include the mitochondrial *p*-benzodiazepine receptor protein (37), caveolin (29), and steroid acute regulatory protein 4 (38). In each case, the direct binding of cholesterol or a sterol analog to the CRM ($V/Lx_{2-4}Yx_{1-4}R/K$) was demonstrated, where x is a neutral or negatively charged amino acid. The C terminus of Nef is an important example of this motif. An additional CRM is found within the viral transmembrane Env protein gp41 (39). These motifs are associated with a steep gradient in local hydrophobicity, which suggests their localization at the membrane interface. Indeed, the positively charged residue (Arg or Lys) may form a salt bridge with the 3-β hydroxyl group of cholesterol.

Previous studies demonstrated that a modification in producer cells is required for Nef to increase viral infectivity (9, 10). In this study, we demonstrated that the wild-type HIV contains more newly synthesized cholesterol. This result is consistent with our previous observation that the wild-type HIV is also more sensitive to cholesterol depletion for its infectivity (25). Why is cholesterol required for HIV infection? Because HIV buds from lipid rafts, the lipid component of viral envelopes should resemble that of DRMs. Thus, cholesterol may enhance fusion by facilitating the formation of the complex between gp120, CD4, and CXCR4. This interpretation is supported by recent reports that cholesterol is essential for (17, 18) and that Nef increases the entry of HIV into cells (40). Importantly, the deletion or point mutation of the cholesterol-binding site in gp41 disrupts significantly the formation of giant cell syncytia (41, 42). These results confirm further the important role of cholesterol in viral replicative cycle.

An attractive model for effects of Nef on viral replication can be drawn (Fig. 6). Our findings suggest that newly synthesized, accessible cholesterol is used primarily to create new virions and indicate that viruses can encode genes, whose protein products are cholesterol transporters, i.e., that viruses can carry their own building blocks. In any case, this notion of proteins carrying lipids for their own needs and of their subservient structures promises to become a major topic in eukaryotic biology. Our findings also open a new perspective for antiviral therapeutics.

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