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Rodent Cells Support Key Functions of the Human Immunodeficiency Virus Type 1 Pathogenicity Factor Nef

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After infection with human immunodeficiency virus (HIV), progression toward immunodeficiency is governed by a complex interplay of viral and host determinants. The viral accessory protein Nef is a key factor for the development of AIDS. Strains of HIV and simian immunodeficiency virus that lack functional *nef* genes either do not induce AIDS or do so only after a significant delay. The validity of a transgenic-small-animal model for de novo infection by HIV will depend on its ability to recapitulate the actions of critical factors of viral pathogenicity, such as Nef. We assessed the ability of rat, mouse, and hamster cells to support key effector functions of Nef. In cell lines from rodents, the subcellular distribution of wild-type HIV type 1 strain SF2 Nef and mutants was comparable to that in human cells. Nef downregulated human CD4 from the cell surface, was associated with p21-activated kinase activity, and enhanced the infectivity of HIV-1 virions. Importantly, these Nef-induced effects, as well as the downregulation of rat CD4 and major histocompatibility complex class I molecules, could also be demonstrated in primary T lymphocytes and macrophages from human CD4-transgenic rats. Thus, HIV-1 Nef exerts key functions in rodent cells. In line with our ongoing efforts to establish a transgenic-rat model of HIV disease, these results indicate that important aspects of viral pathogenesis could be addressed in a transgenic-rodent model permissive for de novo infection and that such a model would be valuable for evaluating the function of Nef in vivo.

The establishment of a small-animal model of human immunodeficiency virus (HIV) pathogenesis would be a boon for the development of novel and more efficient antiviral therapies. Current animal models of HIV disease, including chimpanzees, sooty mangabeys, rhesus macaques, cats, and severe combined immunodeficiency (SCID) mice with fetal human tissues transplanted, have made pertinent contributions to our understanding of lentiviral pathogenesis and to the development and testing of therapeutic strategies. However, these models have significant shortcomings, such as the limited availability and high cost of nonhuman primates, the absence or delayed progression to AIDS, and permissivity only for related retroviruses. Xenotransplant models are informative about some aspects of HIV type 1 (HIV-1) pathogenesis but are technically challenging and present neither a complete range of infected tissues nor the context of an intact immune response.

As an alternative approach, transgenic mice and rats that express the entire HIV-1 proviral genome or subsets of HIV-1 genes have been generated. Although these models develop AIDS-like symptoms (26, 49), they recapitulate only postintegration events or are limited to the study of particular HIV genes. They do not reflect the dynamic interactions occurring during HIV infection in vivo or allow testing of protective vaccine strategies. A new small-animal model that is permissive for HIV infection in the context of an intact immune system would provide an important complement to existing models.

Attempts to generate a fully permissive small-animal model have failed because of several inherent blocks to HIV replication in rodent cells. In mouse NIH 3T3 cells, restrictions to viral entry and transcription were overcome by introducing human CD4 in conjunction with the human chemokine receptors CCR5 and/or CXCR4 (the HIV receptor complex), together with human cyclin T1. The cells were readily susceptible to HIV-1 infection, but few particles were produced, and they were noninfectious (7). Similarly, mice transgenic for human CD4, CCR5, and cyclin T1 were nonpermissive (D. Littman, personal communication). More recently, an additional species-specific restriction factor, the RNA splicing inhibitor p32, was identified in mouse cells (66). p32 appears to be critical for regulating the function of the viral protein Rev in the metabolism of HIV RNA. Overexpression of human p32 significantly increased the production of viral proteins in NIH 3T3 cells, but levels of HIV particles in the supernatant remained very low. This is probably due to additional downstream blocks in the HIV replication cycle, including the assembly and egress of HIV particles (7, 39, 40).

In rat cells, we demonstrated that the major block of HIV replication is at the level of entry and could be overcome by introducing human CD4 and CCR5 (30). These results prompted us to generate rats that are transgenic for the HIV receptor complex. In ex vivo cultures, primary macrophages from human CD4/CCR5-transgenic rats were productively infected by different R5 HIV-1 isolates (29). In contrast, primary T lymphocytes from these animals did not support a full rep-

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lication cycle of HIV and failed to produce progeny virus. Nevertheless, in vivo challenge of these transgenic rats resulted in robust cellular infection and transient, low-level viremia for up to 7 weeks. Once the posttranscriptional block to HIV-1 replication in rat T cells can be overcome, transgenic rats will likely support a more substantial viremia and may serve as a convenient model for studies of aspects of HIV pathogenesis in the context of a dynamic de novo infection.

In humans and primate models, the development of clinical symptoms upon HIV infection and subsequent disease progression depends on a complex interplay of viral and host determinants. The validity of any small-animal model of HIV pathogenesis depends on its ability to accurately reflect the biological activities of those critical factors. One key viral determinant for AIDS pathogenesis is the accessory protein Nef, a myristoylated 27- to 32-kDa protein that is expressed early in the replication cycle. HIV and simian immunodeficiency virus strains lacking functional *nef* genes fail to replicate to high titers in vivo and do not cause clinical symptoms or are associated with significantly delayed disease progression (13, 31, 32). However, *nef*-transgenic mice display an AIDS-like disease (26), indicating that the functions of Nef, at least in the context of germ line expression, are supported in mice. Notably, dynamics of viral-gene expression and pathogenesis during HIV infection may conceivably be quite different from those in *nef*-transgenic models. For other rodent species, including rats and hamsters, similar information is not available.

The exact molecular activities that govern Nef's role in lentiviral pathogenesis are still a matter of debate. Multiple activities of the viral protein are thought necessary to support viral spread in vivo and to facilitate disease progression (reviewed in reference 20). These functions include the removal and degradation of the primary entry receptor CD4 to prevent HIV superinfection and increase particle infectivity (6, 19, 35) and the downregulation of major histocompatibility complex (MHC) class I molecules from the cell surface to interfere with the lysis of productively infected target cells mediated by cytotoxic T cells (10, 54). Nef also triggers several intracellular signaling pathways to raise the activation state of the target cell. In T cells, lowering the activation threshold of the T-cell receptor cascade correlates with the ability of HIV to replicate in primary cells (14, 17, 53, 56, 65). Together with other molecular mechanisms, this effect is triggered by the Nef-mediated activation of the cellular Pak kinase (4, 15, 16, 37, 64). In macrophages, Nef activates the Src kinase Hck (44) and induces the release of soluble CD23 and soluble ICAM, resulting in increased permissivity of T cells for HIV-1 replication (61, 62). Finally, Nef augments the relative infectivity of HIV particles, possibly by modifying the lipid composition of the viral envelope during virus production (43, 59, 67).

Since the biological activity of Nef in rodent cells other than mouse cells was completely unknown, we performed a comprehensive analysis of an established set of functions of Nef in human, mouse, rat, and hamster cells to evaluate the ability of these rodents to support Nef's cardinal role in HIV pathogenesis. Our findings suggest that restriction of Nef function does not present a major obstacle for the development of a small-animal model for HIV infection.

MATERIALS AND METHODS

Plasmids. Constructs encoding wild-type HIV-1_{SF2} Nef and the G2A mutant cloned into pEGFP-N1 (Invitrogen, Karlsruhe, Germany) have been described (33). The EDAA and AXXA mutants were generated by site-directed mutagenesis and subcloning. A three-plasmid lentiviral expression system was used to generate retroviral vector particles that express HIV-1 Nef_{NL4-3}-green fluorescent protein (GFP) fusion proteins under control of the cytomegalovirus intermediate-early promoter. Specifically, the two oligonucleotides MCSs (5′-GATC CCGGGCTAGCTGATCATCTAGACTGCAG-3′) and MCSas (5′-TCGACTG CAGTCTAGATGATCAGCTAGCCCGG-3′) were annealed and directionally inserted into the lentiviral vector pRRL.cPPT.hPGK.GFP.Wpre (provided by Luigi Naldini, Turin, Italy) after digestion with BamHI and SalI to delete the GFP sequence. All chimeras were cloned into the PPT-MCS vector cut with BamHI and XbaI. For construction of the Nef.GFP chimera, the GFP gene was linked by PCR to the 3′ end of NL4-3 *nef*, separated by a stretch encoding a four-glycine spacer, and subcloned by digestion with BamHI and XbaI into the PPT-MCS vector. pCMVΔR8.91 served as the packaging construct (68) and was kindly provided by Didier Trono (Geneva, Switzerland); pVSV-G was kindly provided by Jane Burns (La Jolla, Calif.). A hemagglutinin (HA)-tagged Pak2 expression plasmid was kindly provided by Kalle Saksela (Tampere, Finland) (50). The proviral constructs for HIV-1_{NL4-3} and its counterpart with *nef* deleted have been described (16).

Preparation of viral stocks. Lentiviral stocks were generated by triple transfection of 293T cells by the calcium phosphate method as described previously (30). Two days after transfection, the culture supernatants were harvested and concentrated with Centricon Plus-20 columns (Millipore). The p24 concentrations of concentrated stocks were determined by p24 CA antigen enzyme-linked immunosorbent assay (ELISA) as described previously (45).

Cell lines and transfectants. All cell lines were cultivated under standard conditions in Dulbecco's modified Eagle medium or RPMI 1640 (both from GIBCO, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin-streptomycin, and 1% L-glutamine (both from GIBCO). HeLa, CHO, Rat2, NIH 3T3, SUP-T1, and Nb2 cells were from the American Type Culture Collection (Manassas, Va.). HeLa hCD4/hCCR5 cells, originally from Bruce Chesebro (Rocky Mountain Laboratories, Hamilton, Mo.), were subcloned (provided by Jason Kreisberg, Gladstone Institute of Virology and Immunology, San Francisco, Calif.). Clone H11 was used for all subsequent experiments. CHO hCD4/hCCR5 cells were kindly provided by Mark Goldsmith (Gladstone Institute of Virology and Immunology) and subcloned for high expression by fluorescence-activated cell sorting. Rat2 hCD4/hCCR5 cells were generated by cotransfection of expression plasmids encoding both receptors, selection in G418 and hygromycin B, and subcloning for high expression. NIH 3T3 hCD4/hCCR5/hCycT1 cells were from Dan Littman (Skirball Institute, New York, N.Y.) and were sorted for hCD4/hCCR5 high expressers. SUP-T1 hCCR5 cells and Nb2 hCD4/hCCR5 cells have been described (30).

Primary cells. Cultures of primary rat lymphocytes, rat macrophages, and human lymphocytes from random donors were generated and cultivated as described previously (29). To obtain proliferating lymphocytes, rat splenocyte cultures were activated with concanavalin A (ConA) (Sigma, Munich, Germany) at a final concentration of 4 μg/ml. After overnight incubation, the cells were washed twice with phosphate-buffered saline and resuspended in fresh medium supplemented with 20 nM human recombinant interleukin 2 (IL-2) (a gift from Chiron Corporation). Human peripheral blood mononuclear cells were activated with phytohemagglutinin (Sigma) and IL-2 as described previously (58).

Transfection. Adherent cell lines were plated in 12-well plates and transfected with 1 μg of plasmid DNA; Lipofectamine (Invitrogen) was used for NIH 3T3, HeLa hCD4/hCCR5, and NIH 3T3 hCD4/hCCR5/hCycT1 cells; Lipofectamine 2000 (Invitrogen) was used for CHO, CHO hCD4/hCCR5, and Rat2 hCD4/hCCR5 cells; and Fugene (Roche Molecular Biochemicals, Mannheim, Germany) was used for Rat2 and HeLa cells. The T-cell lines SUP-T1 and Nb2 were transfected by electroporation as described previously (30). Activated primary human peripheral blood mononuclear cells were transiently transfected with the Human T Cell Nucleofector kit (Amaxa Biosystems, Braunschweig, Germany) using 10^7 viable cells, 2 μg of plasmid DNA, and Nucleofector program T-23. Activated primary rat lymphocytes were transfected in a similar fashion using Nucleofector program U-14.

Flow cytometry. Fluorescence-activated cell sorting analyses were performed as described previously (28) using phycoerythrin (PE)-conjugated anti-human CD4 (monoclonal antibody [MAb] Leu-3a), PE-conjugated anti-rat MHC class I (RT1A; MAb OX-18), allophycocyanin (APC)-conjugated anti-rat CD4 (MAb OX-35), PE-conjugated anti-rat CD3 (MAb G4.18), and peridinin chlorophyll protein (PercP)-conjugated anti-rat CD8a (MAb OX-8). All antibodies were

from BD Pharmingen (Heidelberg, Germany). Samples were collected on a FACSCalibur or FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) and analyzed with Cellquest software.

Immunofluorescence microscopy. To visualize the subcellular localization of Nef.GFP fusion proteins, cells were seeded onto glass coverslips and transfected with 1 μg of DNAs of the different constructs. Twenty-four hours after transfection, the cells were fixed with 3% paraformaldehyde–phosphate-buffered saline (10 min at room temperature) and mounted with Histoprime (Histogel, Linaris, Germany). Fluorescence microscopy images were acquired with an Olympus IX 70 microscope with a 60× oil immersion objective equipped with a charge-coupled device camera and processed with analySIS version 3.0 software (Soft Imaging System, Münster, Germany). Final images were processed in Photoshop version 6.0 (Adobe Systems, Mountain View, Calif.).

HIV-1 infectivity assay. The relative infectivity of HIV-1 particles was determined by p24 CA ELISA and a standardized 96-well TZM (JC53BL) blue-cell assay as described previously (63).

Western blot analysis. For Western blot analysis, cell lysates were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. After incubation with primary and secondary antibodies, protein was detected with the SuperSignal pico detection kit (Pierce, Bonn, Germany).

p21-activated kinase assay. Nef-associated Pak activity was assessed by analyzing the autophosphorylation of Pak in an in vitro kinase assay (IVKA) of Nef immunoprecipitates from total cell lysates 18 to 24 h after transfection as described previously (15). Briefly, cells expressing various Nef.GFP fusion proteins were lysed in KEB (137 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 0.5% NP-40, Na_3VO_4) supplemented with protease inhibitors (Roche Molecular Biochemicals). The cleared lysates were immunoprecipitated with anti-GFP antibodies. After intensive washing in KEB, the immunoprecipitates were resuspended in KAB (50 mM HEPES, pH 8, 150 mM NaCl, 5 mM EDTA, 0.02% Triton X-100, 10 mM $MgCl_2$) containing 10 μCi of [γ-^{32}P]ATP (Amersham, Freiburg, Germany) per reaction. After incubation for 5 min, samples were washed, and bound proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography.

RESULTS

The subcellular distribution of HIV-1_{SF2} Nef wild type and mutants is largely preserved in rodent cell lines. The subcellular localization of Nef is a critical factor for its biological activity. Largely mediated by its N-terminal myristoylation, Nef associates with the plasma membrane and intracellular membranes of the host cell. This localization is thought to be vital for virtually all described functions of Nef (20). We compared the subcellular localization of a prototypic Nef from the HIV-1 strain SF2 in transiently transfected Rat2 (rat), NIH 3T3 (mouse), and CHO (hamster) cells to that in human HeLa cells (Fig. 1). Nef.GFP fusion proteins were used throughout the study as a well-established functional homologue of nonfused Nef, allowing the direct comparison of localization studies and functional analyses (22, 33).

In HeLa cells, Nef.GFP was found predominantly in cytoplasmic punctae, possibly reflecting the association of Nef with intracellular transport machineries, and at the plasma membrane and diffusely in the cytoplasm (Fig. 1B), consistent with previous reports (22, 33). As expected, this distribution was entirely dependent on myristoylation. The NefG2A.GFP mutant, in which the myristoyl acceptor glycine at position 2 is replaced by alanine, was exclusively localized diffusely in the cytoplasm (Fig. 1C), resembling the distribution seen for GFP alone (Fig. 1A). Mutation of the PXXP SH3 binding motif in Nef to AXXA did not markedly alter the subcellular localization compared to wild-type Nef (Fig. 1E), although a slight accumulation at perinuclear clusters was observed that was even more pronounced for the NefEDAA mutant (Fig. 1D). The NefEDAA.GFP protein was abundant at the plasma membrane but was also present in large vesicular perinuclear structures, probably reflecting its inability to interact with the endocytic machinery responsible for internalization mediated by the AP2-clathrin pathway (21, 23, 36).

The subcellular distributions of these GFP fusion proteins in various adherent rodent cell lines was strikingly similar to that in human HeLa cells. Nef.GFP was always localized in a punctate pattern and at the plasma membrane in cells of all three species, and this localization was dependent on an intact N-terminal myristoylation signal. The distribution of the AXXA and EDAA mutants of Nef in Rat2, NIH 3T3, and CHO cells was also comparable to that in HeLa cells. Only slight variations in the size of the punctae were sometimes noted (e.g., in CHO cells [Fig. 1Q]). We conclude that the cellular factors that determine the subcellular localization of HIV-1 Nef in human cells are essentially conserved in rodent cells.

HIV-1_{SF2} Nef downregulates human CD4 on adherent and T-cell lines of human, rat, mouse, and hamster origin. Next, we characterized the ability of rodent cells to support well-established functions of Nef. First, we investigated the ability of Nef to efficiently downregulate human CD4, the primary entry receptor for HIV-1, from the cell surface. In cell lines stably expressing human CD4 and CCR5, GFP fusion proteins of wild-type Nef and specific mutants were transiently expressed. Steady-state cell surface levels of human CD4 were determined by flow cytometry and expressed as a percentage of the mean fluorescence intensity (MFI) of GFP-expressing cells 2 days after transfection. NefG2A.GFP and NefEDAA.GFP served as negative controls, as they are both drastically impaired in CD4 downregulation due to their lack of plasma membrane localization or inability to recruit the endocytic machinery, respectively (1, 27, 36). An additional mutant, NefAXXA.GFP, which is defective in binding to SH3 domains, is not impaired in CD4 downregulation in human cells (52).

In HeLa hCD4/hCCR5 cells, expression of Nef.GFP and NefAXXA.GFP reduced cell surface levels of human CD4 by ~90% (Fig. 2A). As expected, the G2A and EDAA mutants failed to markedly reduce CD4 levels. In adherent rodent cell lines, Nef.GFP downregulated human CD4 by 60 to 75% (Fig. 2B, C, and D). These slightly lower efficiencies were not due to a general species restriction, since Nef.GFP downregulated human CD4 to a greater extent in rat Nb2 T cells (~85%) (Fig. 2H) than in human SUP-T1 T cells (~60%) (Fig. 2F). Nef mutants displayed similar patterns of activity in both adherent and T-cell lines of rat, mouse, and hamster origin. The Nef.GFP fusion proteins were stably expressed at comparable levels in all cell lines, as determined by anti-GFP Western blot analysis (Fig. 2E [CHOhCD4/hCCR5 cells] and F [SUP-T1 cells] and data not shown). In summary, the effects of the mutant Nef proteins suggest that the cellular machinery Nef uses to reduce cell surface CD4 levels is conserved in rodents.

Wild-type HIV-1_{SF2} Nef, but not the nonmyristoylated mutant, associates with p21-activated kinase activity in rodent cell lines. We next investigated the ability of Nef to associate with Pak kinase activity as a functional correlate for the induction and modulation of cellular signal transduction. HeLa, NIH 3T3, Rat2, and CHO cells were cotransfected with expression constructs for either Nef.GFP or NefG2A.GFP, together with a construct for epitope-tagged Pak2. To determine if Nef could associate with endogenous Pak activity, an exper-

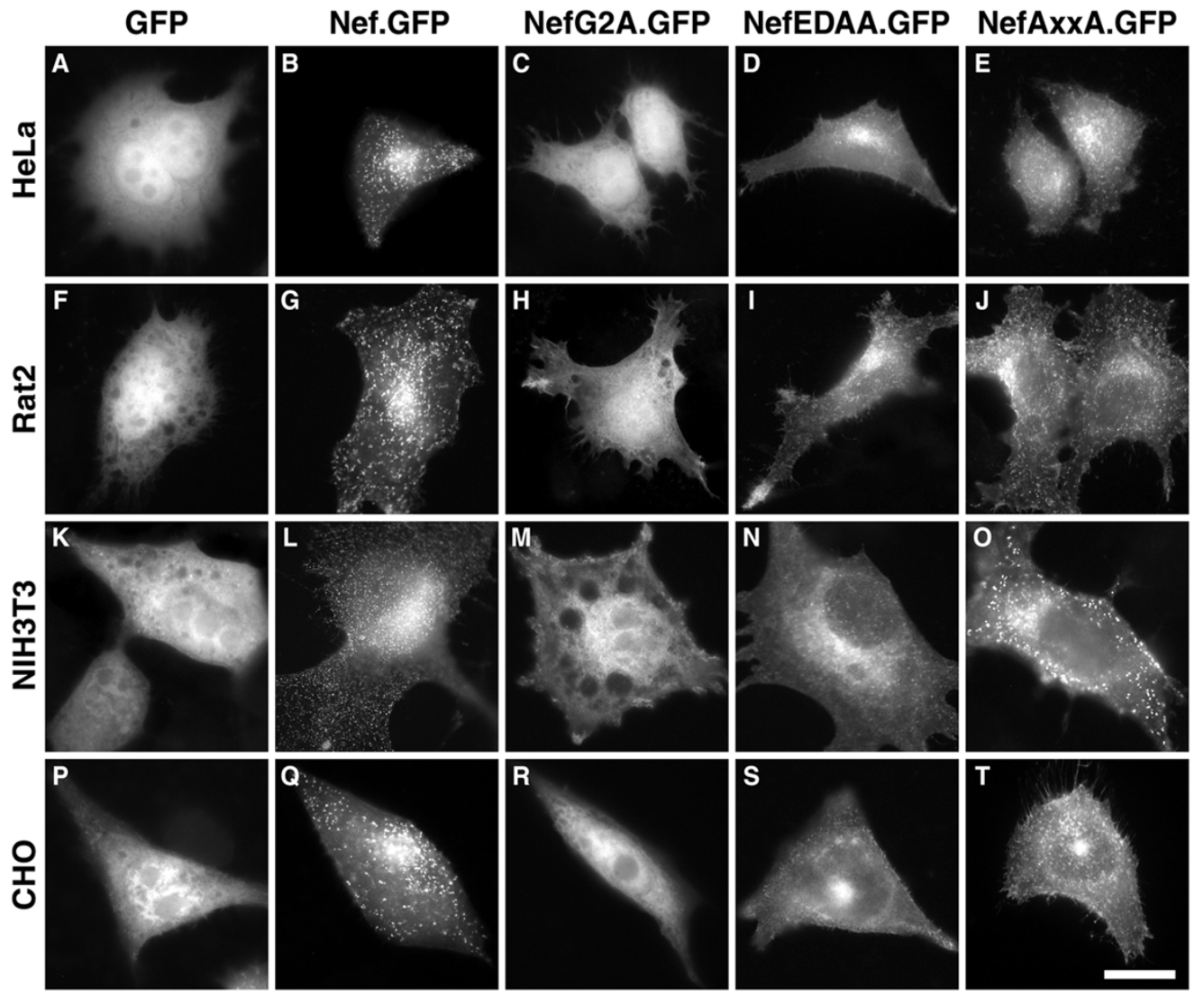

FIG. 1. The subcellular distribution of HIV-1_{SF2} Nef wild type and mutants is largely preserved in rodent and human cell lines. Human and rodent cells constitutively expressing human CD4 and CCR5 were plated on coverslips and transfected with expression constructs encoding the indicated Nef.GFP fusion proteins or GFP alone. One day after transfection, the cells were fixed with paraformaldehyde, and digital images of GFP fluorescence were acquired on an Olympus IX 70 microscope. Scale bar, 20 μm.

imental condition with expression of Nef.GFP alone was included. Anti-GFP immunoprecipitates were analyzed for Pak activity with an IVKA for autophosphorylated kinase (Fig. 3, top, IVKA). In parallel, expression of Nef.GFP or NefG2A.GFP was analyzed in cell lysates by anti-GFP Western blotting (Fig. 3, bottom, WB), which demonstrated comparable expression levels of the Nef.GFP constructs in the various cell lines.

Pak2 activity was readily detected by the presence of an ~66-kDa phosphorylated protein in IVKA reactions of anti-GFP immunoprecipitates from HeLa cells expressing Nef.GFP, but not NefG2A.GFP (Fig. 3, compare lanes 6 and 4). The slower migration of phosphorylated Pak2 (usually at 62 kDa) was due to the presence of the HA epitope tag. In the absence of coexpressed Pak2, Nef.GFP associated with endogenous Pak activity in HeLa cell lysates (Fig. 3, lane 5). In NIH 3T3 (Fig. 3, lanes 1 to 3), CHO (lanes 7 to 9), and Rat2 (lanes 10 to 12) cells, Nef.GFP also associated specifically with increased activity of heterologously expressed Pak2, as well as endogenous Pak. As a specificity control, the nonmyristoylated G2A mutant showed no signal or a significantly lower activity than wild-type Nef. When these data were taken together, Nef.GFP was found to associate efficiently with exogenous Pak2, as well as endogenous Pak activity, in human and rodent cells.

Nef enhances the infectivity of HIV-1 virions produced in rodent cell lines and primary rat macrophages. In human cells, Nef enhances HIV-1 particle infectivity 3- to 10-fold during virus production (2, 47). To determine if rodent cells support this function, CHO, Rat2, and human HeLa control cells were infected with vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 NL4-3wt and its *nef*-deficient counterpart, HIV-1 NL4-3Δ*nef*. Culture supernatants containing NL4-3wt

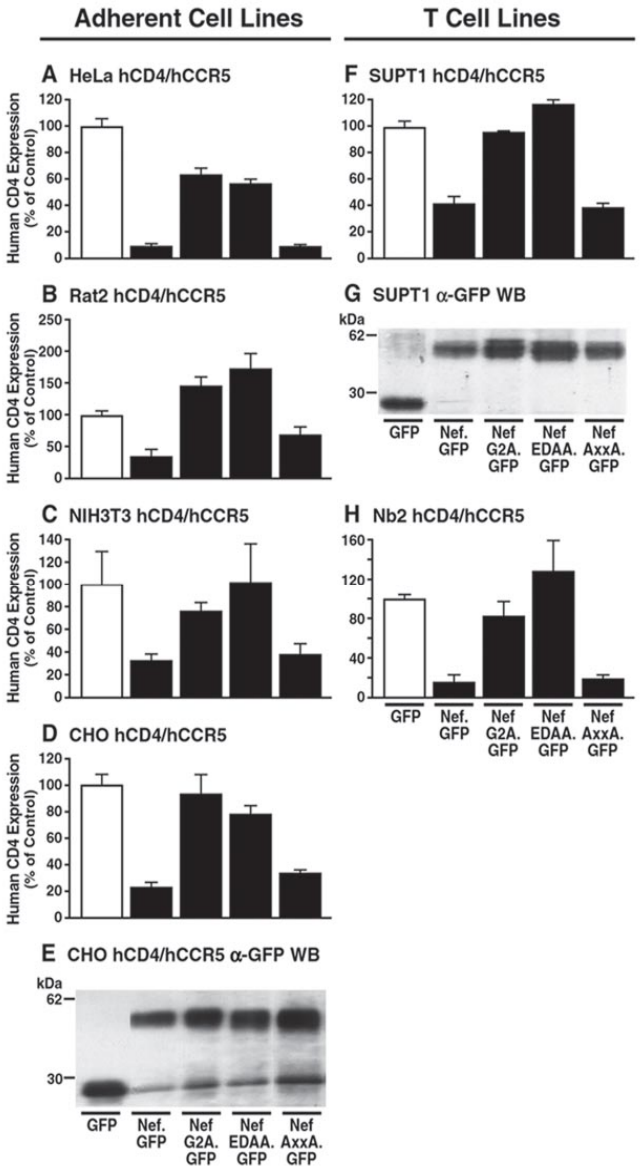

FIG. 2. HIV-1_{SF2} Nef efficiently downregulates human CD4 in adherent and T-cell lines of human, rat, mouse, and hamster origin. Human and rodent adherent cells (A to E) and T-cell lines (F to H) coexpressing human CD4 and CCR5 were transfected with expression constructs encoding wild-type Nef and Nef mutants fused to GFP. Two days after transfection, steady-state cell surface levels of human CD4 in cells with medium- and high-level expression of GFP were determined by flow cytometry. Values are expressed as percentages of the MFI in control cells expressing GFP alone. The values shown are the arithmetic means (plus standard deviations) of triplicates from one representative experiment out of three to eight independent experiments performed. (E and G) Anti-GFP Western blot analyses of lysates from hamster CHOhCD4/hCCR5 cells and SUPT-1 cells, respectively, transfected with expression plasmids for the individual Nef.GFP fusion proteins.

and NL4-3Δ*nef* particles released from these three cell types were harvested 2 days after infection. NIH 3T3 cells released almost no virions and could not be analyzed in this assay (references 7 and 40 and data not shown). The concentration of viral particles in supernatants was quantified by p24 CA ELISA, and the relative infectivity of particles was determined in a single round of replication on the TZM reporter cell line, in which productive infection by HIV triggers expression from an HIV-1 long-terminal-repeat-driven β-galactosidase gene (34, 63). Infections were carried out with a normalized input of 1 ng of p24 per 96-well plate, and the relative virion infectivity was plotted as the number of infected cells per nanogram of p24. As expected, NL4-3wt particles produced by HeLa cells were approximately sixfold more infectious than NL4-3Δ*nef* virions (Fig. 4A). Nef increased virion infectivity about threefold in CHO cells and sixfold in Rat2 cells (Fig. 4A). Importantly, the infectivity of wild-type virions produced in hamster and rat cell lines was similar to or even slightly greater than that of particles produced in HeLa cells, indicating that cells from these rodents are not generally impaired in the production of infectious HIV-1. HIV-1_{NL4-3} virions produced by rat primary spleen macrophages also supported Nef.mediated enhancement of infectivity (Fig. 4B). Notably, the absolute levels of virion infectivity differed significantly between macrophage cultures from the two animals analyzed. Taken together, hamster and rat cell lines, as well as primary rat macrophages, supported the enhancement of virion infectivity by HIV-1 Nef.

Primary lymphocytes from human CD4-transgenic rats support Nef.mediated downregulation of human and rat CD4 and a Nef.associated signaling function. Next, we investigated whether primary cells from human CD4-transgenic rats also supported additional critical activities of HIV-1 Nef. ConA–IL-2-activated lymphocytes from the spleens of the rats were transiently transfected with constructs encoding Nef.GFP fusion proteins by nucleofection. The cell surface expression of rat and human CD4 on GFP-positive CD4 T lymphocytes was analyzed by four-color flow cytometry 1 day after transfection. CD4 T cells were identified as viable $CD3^+$ $CD8^-$ lymphocytes (Fig. 5A and B, R1 and R2 gates), and the expression levels, reflected by the MFI, of human and rat CD4 were determined (Fig. 5C to E, R3 gate) and quantified (Fig. 5F and G). Nef.GFP and NefAXXA.GFP efficiently downregulated both endogenous rat CD4 and transgenically expressed human CD4 in primary CD4 T cells. The degree of downregulation was clearly dependent on the expression level of Nef.GFP (Fig. 5C and E), was completely abrogated by mutation of the ED motif (Fig. 5D), and was comparable to that of Nef.GFP in rodent and human cell lines.

HIV-1 Nef also downregulates human MHC class I molecules, specifically the HLA-A2 allele, from the cell surface (8, 10, 54). Because of the fundamentally different organization of the MHC class I complex in rodents, it is not possible to define an ortholog of HLA-A2 in these species (25). Using an experimental setup for transient transfection of primary rat T cells identical to that described above, we determined the overall level of rat MHC class I cell surface expression by using the broadly reactive monoclonal antibody OX-18 to detect a nonpolymorphic determinant of the rat MHC class I complex (RT1A). A modest, but significant, effect on the cell surface expression levels of rat MHC class I by Nef.GFP and NefEDAA.GFP (22 and 26% downregulation, respectively), relative to the nonfunctional NefAXXA.GFP mutant, could be detected (Fig. 6). This indicates that primary rat cells can, in principle, support the downmodulation of MHC class I by HIV-1 Nef.

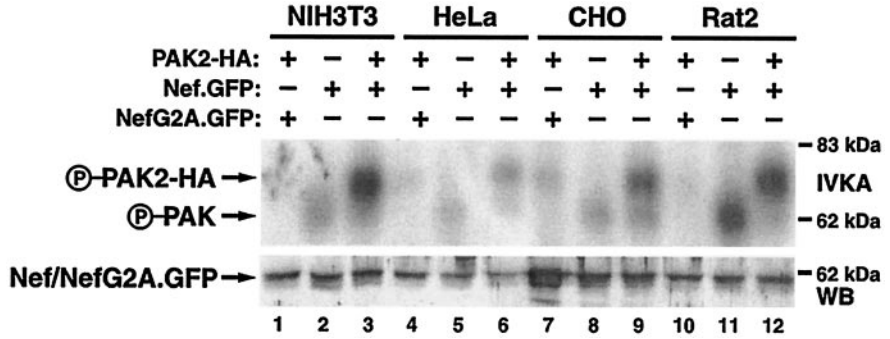

FIG. 3. HIV-1_{SF2} Nef wild type, but not the nonmyristoylated G2A mutant, is associated with robust Pak activity in rodent cell lines. Human and rodent cell lines were transfected with (+) expression constructs encoding Nef.GFP alone, Nef.GFP and Pak2-HA, or NefG2A.GFP and Pak2-HA. One day after transfection, anti-GFP immunoprecipitates from cell lysates were analyzed in an IVKA. The autoradiogram (top) and the corresponding anti-GFP Western blot (WB) (bottom) of the input samples are shown. This experiment is representative of three independent experiments. Encircled P, ^{32}P.

Next, we sought to determine the ability of primary rat T cells to support Nef.mediated signaling functions. The IVKA for Pak autophosphorylation described above was performed on mitogen–IL-2-activated lymphocytes from a human donor and from two rats 1 day after transfection. Nef.GFP, but not the mutant defective in the PXXP motif (NefAXXA.GFP), was associated with endogenous Pak activity in these primary cells (Fig. 7). The frequency of transfected cells and the expression levels of both fusion proteins were comparable, as determined by flow cytometry (see the legend to Fig. 7).

Expression of Nef downregulates CD4 and induces its accumulation in vesicular compartments in primary macrophages from transgenic rats. Besides CD4 T cells, macrophages are the most important target cells for productive HIV-1 infection. Macrophages in human CD4/CCR5-transgenic rats express the HIV receptor complex and are permissive for HIV infection (29). To specifically address the functional consequences of Nef expression in these cells, primary macrophages from CD4-transgenic rats were transduced with VSV-G-pseudotyped lentiviral constructs expressing either HIV-1_{NL4-3} Nef.GFP or NefG2A.GFP fusion protein. Four days after transduction, the expression and subcellular localization of human CD4 and Nef fusion proteins was determined on fixed and permeabilized cells by immunofluorescence mi-

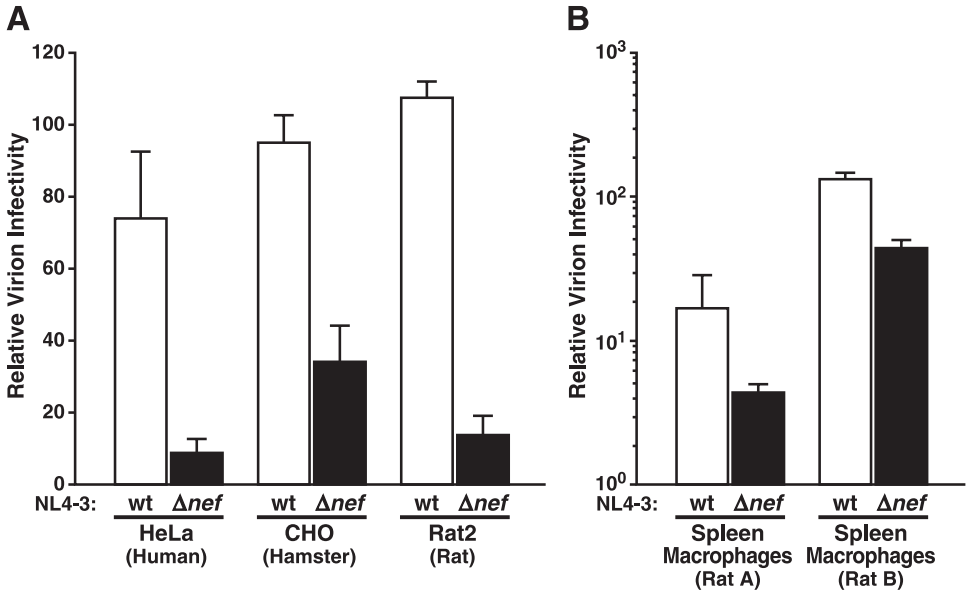

FIG. 4. HIV-1_{SF2} Nef enhances the infectivity of HIV-1 produced in human and rodent cell lines (A) and primary rat macrophages (B). Cells were infected with stocks of HIV-1 NL4-3 wild type (wt) or NL4-3Δ*nef* (Δ*nef*) pseudotyped with VSV-G. Three days after infection, the supernatants were harvested, and the concentrations of the p24 CA antigen were determined (HeLa, NL4-3 wild type, 1,250 ng/ml; NL4-3Δ*nef*, 1,200 ng/ml; CHO, NL4-3 wild type, 62 ng/ml; NL4-3Δ*nef*, 165 ng/ml; Rat2, NL4-3 wild type, 14 ng/ml; NL4-3Δ*nef*, 10 ng/ml. Macrophages: Rat A, NL4-3 wild type, 14 ng/ml; NL4-3Δ*nef*, 16 ng/ml; Rat B, NL4-3 wild type, 22 ng/ml; NL4-3Δ*nef*, 21 ng/ml). The relative infectivity of viral supernatants (corresponding to 1 ng of p24 CA) was determined in a standardized 96-well blue-cell assay on TZM indicator cells, carrying a long-terminal-repeat-driven β-galactosidase gene. The values for relative virion infectivity (number of blue cells/nanogram of p24) represent the arithmetic mean of quadruplicates. The values in panel B are presented on a logarithmic scale. The error bars indicate standard deviations.

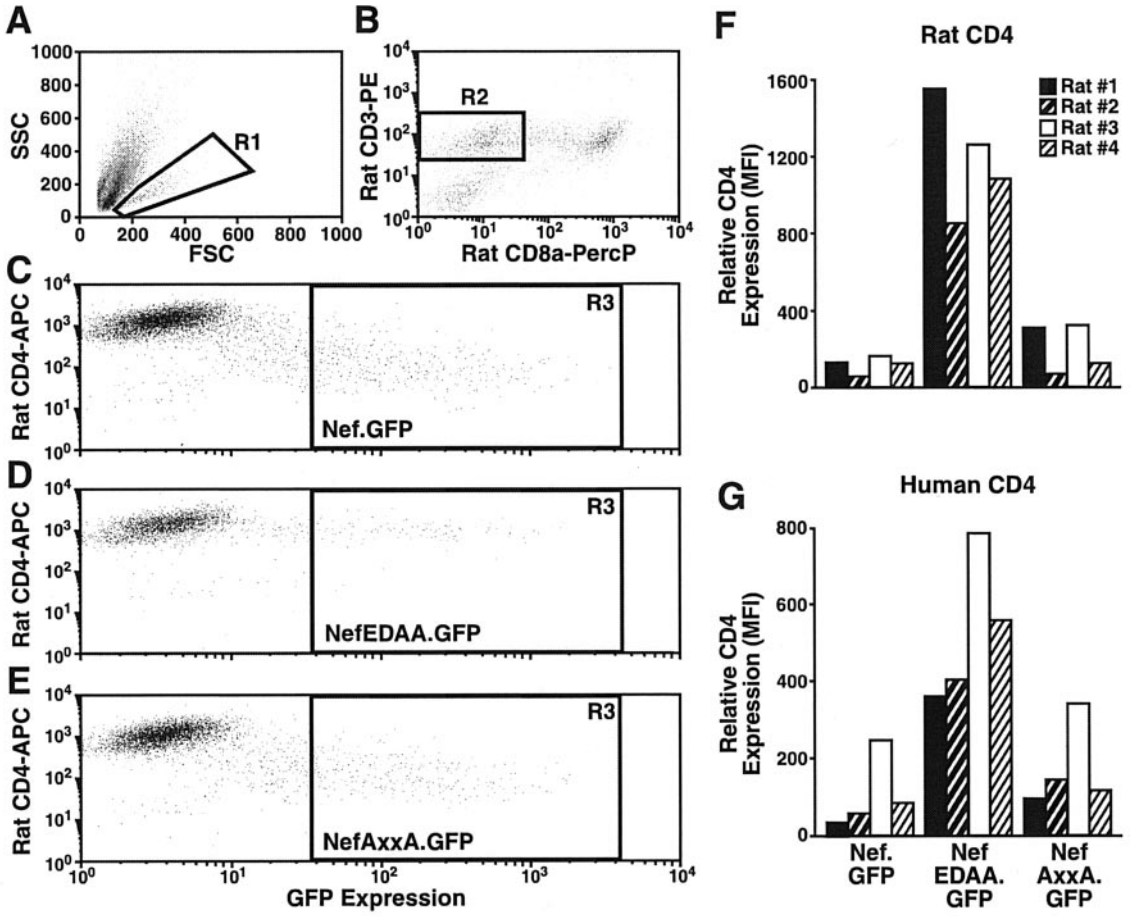

FIG. 5. HIV-1_{SF2} Nef efficiently downregulates rat and human CD4 on primary rat T cells. ConA–IL-2-activated rat splenocytes were transfected by nucleofection with constructs encoding either Nef.GFP (C), NefEDAA.GFP (D), or NefAXXA.GFP (E). One day after transfection, cell surface expression of rat or human CD4 on CD4 T cells was analyzed by flow cytometry. CD4 T cells were identified as viable (A, R1 gate) rat $CD3^+$ $CD8^-$ (B, R2 gate) lymphocytes. Cell-surface levels of rat (F) or human (G) CD4 in GFP-expressing cells in the R3 gate (C to E) were quantified by flow cytometry as the MFI. The R3 gate was arbitrarily set to include cells with medium and high expression levels. Absolute values of splenocyte samples from four rats are shown. One of two comparable experiments is shown. SSC, side scatter; FSC, forward scatter; APC, antigen-presenting cells.

croscopy. Compared to macrophages expressing the nonmyristoylated GZA mutant, Nef.GFP expression resulted in a significant reduction of surface-exposed human CD4 (Fig. 8, compare left with middle and right images). Interestingly, this loss from the cell surface coincided with an accumulation of human CD4, as well as Nef.GFP, in smaller and larger cytoplasmic vesicles (Fig. 8). Notably, larger vesicular structures containing human CD4 were also seen in macrophages expressing the G2A mutant, suggesting that this compartment, possibly representing CD63-positive multivesicular bodies, may be utilized during the physiological life cycle of CD4 molecules.

DISCUSSION

This study demonstrates that cells of rat, mouse, and hamster origin support cardinal effector functions of the HIV pathogenicity factor Nef. Several functional consequences of Nef expression that are well established in human cells were readily detected in rodent cell lines and primary T cells and macrophages from transgenic rats. The downmodulation of surface-exposed CD4 and MHC class I molecules by Nef, the ability of Nef to associate with activity of the cellular signaling effector Pak, and the positive effect of Nef on HIV-1 particle infectivity were recapitulated in rodent cell lines and in primary rat cells. These findings suggest that Nef will not be a limiting factor in the development of a small-animal model of HIV disease. Furthermore, such an experimentally accessible model may facilitate the elucidation of Nef's role in the context of a dynamic in vivo infection.

To address the integrity of Nef's pathogenic potential in rodent cells, we examined individual characteristics and functions of Nef that reflect the diversity of Nef phenotypes and are considered to be important in human cells. As judged from immunofluorescence analyses, the overall subcellular distribution and membrane association of Nef found in human cells were preserved in rodent cells. This is particularly critical because the correct subcellular localization of Nef, in particular at the plasma membrane, appears to be a prerequisite for virtually all of its known activities (20).

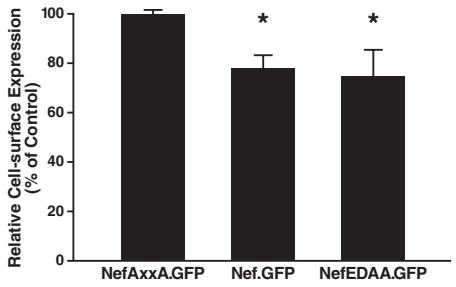

FIG. 6. HIV-1_{SF2} Nef modestly downregulates rat MHC class I on primary rat CD4 T cells. ConA–IL-2-blasted rat splenocytes were transfected by Amaxa nucleofection with constructs encoding either NefAXXA.GFP, Nef.GFP, or NefEDAA.GFP. One day posttransfection, cell surface expression of a nonpolymorphic determinant (RT1A) of rat MHC class I was analyzed by multicolor flow cytometry. Viable CD4 T cells were identified as described in the legend to Fig. 5. The MFI of cells expressing medium to high levels of Nef.GFP fusion proteins was determined, and values for the nonfunctional NefAXXA.GFP mutant were set at 100%. The arithmetic means and standard deviations of values obtained from splenocyte cultures from four rats in two independent experiments are shown. The statistical significance (*) of the differences in MHC I cell surface levels relative to cells expressing NefAXXA.GFP was verified by Student's *t* test ($P = 0.0002$ for Nef.GFP and $P = 0.0028$ for NefEDAA.GFP).

Nef was clearly capable of reducing cell surface levels of human CD4 on adherent and T-cell lines from rodents, confirming results in mouse cell lines (3, 12) and extending these findings to rat and hamster cells. Analysis of Nef mutants demonstrated the importance of the myristoylation signal and the C-terminal flexible loop for this function. Thus, Nef.mediated CD4 downregulation is not species specific, suggesting that the cellular machineries required by Nef are conserved in rodents. This is not unexpected, since Nef exploits a central transport pathway of eukaryotic cells by triggering CD4 endocytosis via clathrin-coated vesicles and subsequent lysosomal degradation (24). The efficient downregulation of CD4 appears to be of great importance for HIV, since Env and Vpu also exert this function (41). The loss of cell surface CD4 is believed to prevent viral superinfection and to counteract negative effects of CD4 on particle release and virion infectivity (6, 35, 51).

Downregulation of endogenous CD4 has been demonstrated in T cells from *nef*-transgenic mice (9, 26, 57). We found that rat CD4 was also efficiently downregulated after transient expression of Nef in primary T cells from transgenic rats. This function may be especially relevant for the HIV-induced impairment of CD4 T helper cell and immune responses in an infectible-small-animal model. An additional immunomodulatory function of Nef is linked to its ability to downregulate cell surface MHC class I molecules, specifically HLA-A2, thereby facilitating the escape of infected human CD4 T cells from killing mediated by cytotoxic T lymphocytes (10). Recently, Nef was suggested to be less efficient in reducing cell surface levels of heterologously expressed mouse MHC class I on HeLa cells than of endogenous human MHC class I and to be impaired in its ability to downregulate class I molecules from mouse endothelial cells (18).

We found that cell surface-exposed rat MHC class I molecules, detected by the nonpolymorphic RT1A determinant (MAb OX-18), were downmodulated by 22 to 26% in the presence of Nef.GFP or NefEDAA.GFP in primary CD4 T cells from rats. This shows that, in principle, the cell surface expression of rat MHC class I molecules can be affected by Nef. Comparable analyses in primary human CD4 T cells using an HLA-A2-specific antibody show a 20 to 50% downregulation (O. T. Keppler and O. T. Fackler, unpublished results). The rather modest degree of downregulation in rat cells has to be interpreted cautiously, because orthologs of human HLA-A2 cannot be defined in the MHC class I complexes of rodents (25). Thus, in-depth analyses of the effects of Nef on rodent MHC class I molecules are currently precluded.

Nef supported the enhancement of virion infectivity in rodent cell lines and, importantly, in primary rat macrophages. CD4-dependent (35) and -independent mechanisms (46) have been implicated in this infectivity phenotype, and we did not attempt to assess their relative contributions. Since Nef increased HIV infectivity to similar extents in rodent and human cells, both mechanisms are likely to be conserved in rodents. CD4-independent enhancement of virion infectivity is achieved by the targeting of assembly intermediates to lipid raft microdomains (67). Our recent evidence suggests that this targeting is mediated by the interaction of Nef with Gag Pol (11). Thus, the conservation of this function may explain not only the ability of Nef to enhance virion infectivity in rat and hamster cells, but also the production of infectious HIV particles from these species in general.

In addition to blocks described for the function of the HIV proteins Tat and Rev, another species-specific restriction factor, APOBEC3G, has recently been identified (38). This antiviral, virion-encapsidated cellular protein renders HIV-1 particles noninfectious by deaminating minus-strand reverse-transcript cytosines to uracils (55). In human cells, the HIV-1 protein Vif preserves infectivity by interfering with the translation of APOBEC3G, inhibiting its incorporation into virions and promoting its proteasomal degradation (38, 60). However, Vif cannot efficiently form a complex with mouse APOBEC3G

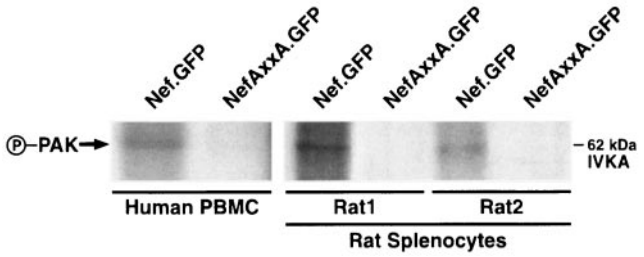

FIG. 7. HIV-1_{SF2} Nef wild type, but not the PXXP mutant, is associated with Pak activity in primary rat and human T cells. Phytohemagglutinin–IL-2-activated human peripheral blood mononuclear cells from one donor and ConA–IL-2-activated rat splenocytes from two rats were transfected by nucleofection with constructs encoding either Nef.GFP or the PXXP mutant (NefAXXA.GFP). One day after transfection, anti-GFP immunoprecipitates from cell lysates were analyzed in an IVKA. Levels of GFP-positive cells among viable cells were comparable for the respective Nef.GFP and NefAXXA.GFP samples (human peripheral blood mononuclear cells [PBMC], 23 and 28% GFP positive; Rat1 splenocytes, 43 and 34% GFP positive; Rat2 splenocytes, 33 and 35% GFP positive). The autoradiogram is shown. The band corresponding to ^{32}P-Pak (62 kDa) is indicated on the right. Encircled P, ^{32}P.

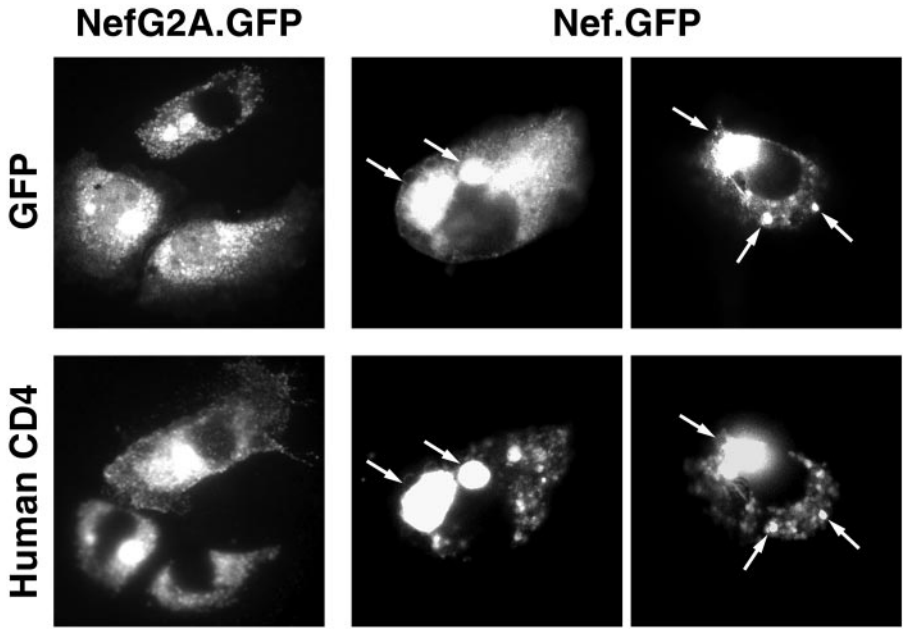

FIG. 8. HIV-1_{NL4-3} Nef downregulates human CD4 from the cell surfaces of primary macrophages from transgenic rats, and both molecules colocalize in intracellular vesicles. Primary spleen macrophages from a human CD4-transgenic rat were cultured on coverslips and transduced with VSV-G-pseudotyped lentiviral vectors encoding either Nef.GFP or NefG2A.GFP. Five days after transduction, the cells were fixed with paraformaldehyde, permeabilized with saponin, and stained with a PE-conjugated antibody to human CD4. Images for GFP and PE fluorescence were acquired with an Olympus IX 70 microscope. Human CD4 and Nef.GFP colocalized in large intracellular vesicles (arrows).

and, consequently, cannot prevent the encapsidation of APOBEC3G. This species-specific restriction was also described for hamster APOBEC3G but, interestingly, not for rat APOBEC3G (38). Consequently, it was assumed that mouse and hamster cells interfere with the release of infectious wild-type HIV particles by the action of this antiviral factor. Our experimental data do not support this conclusion for hamster cells. CHO cells, like Rat2 cells and primary rat macrophages, secreted wild-type virions that were as infectious as virions derived from human cells. This indicates that, at least in this hamster cell line, APOBEC3G expression does not significantly interfere with virion infectivity. Thus, based on these functional data, APOBEC3G or other isoforms of APOBEC3 probably do not represent an obstacle for the development of an infectible transgenic-small-animal model of HIV infection in rats and hamsters.

Finally, the ability of Nef to induce signal transduction was addressed using the association of Nef with Pak activity as a marker. Cells from all rodent species supported this activity of Nef. Nef associated with overexpressed Pak2 activity and with an endogenous kinase activity, resulting in the phosphorylation of a 62-kDa protein. Since Pak kinases are well conserved in rodent cells (5), this phosphoprotein is likely to be autophosphorylated rodent Pak. In line with reports demonstrating activation of the T-cell receptor cascade in mouse cells by Nef in vitro and in vivo (12, 26), these data suggest that T-cell activation by Nef is readily supported in rodents. In an infectible-small-animal model, this may be particularly relevant for the transmission of HIV-1 from immature dendritic cells to T cells, which requires Nef and correlates with its ability to activate Pak (17, 42, 48).

The present study demonstrates that key functions of Nef can be recapitulated in rodent cells, including rat and hamster cells, which have never been addressed in this context. First, the subcellular distribution of HIV-1 Nef, which is an important and well-established prerequisite for its biological activities in human cells, was preserved in mouse, rat, and hamster cells. This demonstrates that the cellular machineries that govern Nef's localization are active in all of these rodents. Second, the positive effect of HIV-1 Nef on particle infectivity for virus derived from rat and hamster cells, including primary rat macrophages, was demonstrated for the first time. This finding is of great relevance for studies of Nef biology in the context of virus replication in transgenic rats or hamsters that are being developed as fully permissive models for de novo infection by HIV. Third, the Nef.associated signaling function (Nef.associated Pak autophosphorylation) in rat cell lines, hamster cell lines, and, importantly, primary rat T cells in a transient-expression context was demonstrated for the first time. Fourth, the Nef. mediated downmodulation of transgenically expressed human CD4, endogenous rat CD4, and rat MHC class I in primary CD4 T cells and macrophages from transgenic rats was shown. This finding supports the prospect that a fully susceptible transgenic-rodent model will allow the scientific community to study the role of these Nef effects in superinfection and immune evasion in vivo.

Our results are in line with the finding that expression of Nef is sufficient for the induction of an AIDS-like disease in a transgenic-mouse model (26). Similarly, pathologies observed in rats transgenic for HIV-1 proviruses may be attributed, at least in part, to the action of Nef (49). The present study further extends these observations by demonstrating that a wide range of specific Nef activities can occur in a transient-expression context relevant for a dynamic HIV infection. We

conclude that, in clear contrast to other regulatory and accessory gene products of HIV, such as Tat, Rev, and Vif, no major species barrier exists for Nef in rodents.

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