Nef-induced Alteration of the Early/Recycling Endosomal Compartment Correlates with Enhancement of HIV-1 Infectivity*

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The HIV-1 Nef protein recognizes preferentially AP-1 and AP-3 adaptor complexes, stabilizing their association with endosomal membranes. These findings led us to hypothesize a general impact of this viral protein on the endosomal system. Here, we have shown that Nef specifically disturbs the morphology of the early/recycling compartment, inducing a redistribution of early endosomal markers and a shortening of the tubular recycling endosomal structures. Furthermore, Nef modulates the trafficking of the transferrin receptor (TfR), the prototypical recycling surface protein, indicating that it also disturbs the function of this compartment. Nef reduces the rate of recycling of TfR to the plasma membrane, causing TfR to accumulate in early endosomes and reducing its expression at the cell surface. These effects depend on the leucine-based motif of Nef, which is required for the membrane stabilization of AP-1 and AP-3 complexes. Since we show that this motif is also required for the full infectivity of HIV-1 virions, these results indicate that the positive influence of Nef on viral infectivity may be related to its general effects on early/recycling endosomal compartments.

Trafﬁcking of membrane proteins is governed by a regulated machinery that involves the vesicular transport of proteins throughout different intracellular compartments. One major regulatory mechanism is related to the function of the adaptor protein (AP) complexes that assemble on donor membranes of the endocytic pathway to form transport vesicles (for review, see Ref. 1). The sorting of transmembrane proteins into these vesicles requires the recognition by the AP complexes of specific tyrosine- or leucine-based motifs contained within the cytoplasmic domains of cargo proteins (2). Four different types of heterotetrameric AP complexes (AP-1–AP-4) have been identiﬁed (3). AP-2 is speciﬁcally involved in the formation of clathrin-coated vesicles at the plasma membrane, whereas AP-1 and AP-3 mediate the formation of clathrin-coated vesicles at the levels of the trans-Golgi network (TGN) and endosomes. The function of AP-4 is less well documented, but it regulates formation of non-clathrin-coated vesicles at the TGN. The association of the AP-1, AP-3, and AP-4 complexes with TGN and endosomal membranes is regulated by ARF-ribosylation factor 1 (ARF1).

The Nef protein of HIV-1 is a 27-kDa protein that associates with the cell membranes through N-terminal myristoylation and is abundantly produced shortly after virus infection (for review, see Refs. 4 and 5). Nef is an essential factor in vivo for efﬁcient viral replication and pathogenesis. In vitro, Nef also facilitates virus replication and enhances the infectivity of virions. Although the positive inﬂuence of Nef on viral replication and infectivity may be multifactorial, genetic evidence suggests a relationship between these virological effects and the ability of Nef to modulate the cell surface expression of multiple membrane-associated proteins. In addition to CD4 and major histocompatibility complex class I (MHC-I) molecules (6–8), the list of membrane proteins in which intracellular trafﬁcking is affected by Nef now includes MHC class II (MHC-II) molecules, the co-stimulatory CD28 molecule, and the lectin DC-SIGN (9–11). These alterations likely promote an immune evasion response of infected cells and enhance the spread of viruses within the host during the natural course of HIV infection (12–15).

Mechanistically, these observations indicate that Nef exerts a general inﬂuence on the intracellular trafﬁcking of membrane proteins. Although the molecular basis of this general effect is not fully understood, it is now evident that it relates to the ability of Nef to interact directly with vesicle coat components involved in the vesicular transport throughout the endocytic pathway, including the clathrin-associated AP complexes. The HIV-1 Nef protein recognizes preferentially AP-1 and AP-3 complexes (16–20) and selectively stabilizes the association of these complexes on endosomal membranes by an ARF1-inde-

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∥The abbreviations used are: AP, adaptor protein; HIV, human immunodeficiency virus; ARF1, ADP-ribosylation factor 1; Tf, transferrin; TfR, transferrin receptor; TGN, trans-Golgi network; GFP, green fluorescent protein; EEA1, early endosome antigen 1; EGF, epidermal growth factor; EGF-TxR, Texas Red-conjugated EGF; EGFR, epidermal growth factor receptor; ERC, endosomal recycling compartment; PE, phycoerythrin; MHC, major histocompatibility complex; Ab, antibody; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Cy, cyanin; WT, wild type.
Lipofectamine (Invitrogen) using 0.5 μl of plasmid given by Dr. N. Landau, La Jolla, CA) used for viral infectivity assays. FITC, BD Biosciences; anti-EEA1 mAb (Transduction Laboratories); anti-TfR, DF1513 anti-CD71 monoclonal antibody (mAb) (Sigma) or Tf-Alexa-594, Tf-Alexa-546, Tf-Alexa-488, and Texas Red-YOYO, NY). GFP-Rme1 expression plasmid was a gift from Dr. F. R. Maxfield (New York, NY). Reagents and Antibodies—Holotransferrin (Tf) was obtained from Sigma, and Tf-Alexa-594, Tf-Alexa-546, Tf-Alexa-488, and Texas Red-conjugated epidermal growth factor (EGF-TxR) were obtained from Molecular Probes (Eugene, OR). Mouse IgG1 anti-CD8, 1H10-15; anti-apoptosis-associated anti-TIR, DF5153 anti-CD71 monoclonal antibody (mAb) (Sigma) or fluorescein-isothiocyanate (FITC)-conjugated anti-TIR mAb (CD71- FITC, BD Biosciences); anti-EA1A1 antibody (Transduction Laboratories); anti-CD8-α, Leu 2A mAb (BD Biosciences), CD8-FITC and CD8-EDC mAbs (Coulter Coultronics); goat anti-Nef polyclonal antibody (23); and anti-p24 mAb (22). Secondary antibodies used in immunofluorescence were from Jackson ImmunoResearch. Anti-Nef polyclonal antibody, anti-CD8-α (Santa Cruz Biotechnology), and anti-GFP (Roche Applied Science) mAbs were used for Western blotting. Secondary horseradish peroxidase-conjugated anti-IgG were from Dako. Cell Lines and Transfections—HeLa and CEM cells were grown respectively in Dulbecco's modified Eagle's medium or in RPMI medium with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). CD4-positive HeLa cells (clone P4.R5, provided by Dr. N. Landau, La Jolla, CA) used for viral infectivity assays were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 100 μg/ml G418. Cells were transfected either by electroporation with 12 μg of plasmid as described above (17) or with Lipofectamine (Invitrogen) using 0.5 μl of plasmid as recommended by the manufacturer.

Viral Infections—Virions were produced by transient transfection of 293T cells with proviral plasmid as described (22), and the concentration of p24 antigen in viral stocks was measured by a quantitative enzyme-linked immunosorbent assay (Abbott Laboratories). Virions were used to infect HeLa-CD4 (clone 1022) cells, and infected cells were subjected 24 h later to Tf uptake as described below. Viral infectivity was determined using an infectious center assay (22). Serial dilutions of viral stock were used to infect in duplicate HeLa-CD4 cells (P4 R5 cells), containing the LACZ indicator under the control of the HIV-long terminal repeat (LTR). After 1 or 2 days at 37°C, the cells were fixed with 1% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline (PBS) and stained in a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution for 4–8 h. Infectivity was assayed by counting the blue-stained, HIV-infected foci. The ratio of blue cells/ng of p24 antigen for each viral stock was normalized to a value of 100% infectivity.

Indirect Immunofluorescence—HeLa cells spread on glass coverslips in 24-well plates (8 × 104 cells/well) were stained for immunofluorescence as described (17). Cells were fixed in 4% paraformaldehyde (Sigma) in PBS, quenched for 10 min with 0.1 M glycine in PBS, and permeabilized with 0.1% Triton-X100 (Sigma) in PBS. Cells were then incubated successively for 30 min at room temperature with the primary and secondary antibodies. For detection of the CD8-Nef chimeras, the cells were blocked for 20 min with 10% normal mouse serum in PBS and stained for 30 min with CD8-FITC mAb. Coverslips were washed and mounted on slides using Immuno-fluor mounting medium (ICN). Confocal microscopy was performed with a Bio-Rad MRC1000 or a Leica DMI2620 instrument. Images were processed using Adobe Photoshop software. Where indicated, the intensity of fluorescence was quantified by high resolution measurements using the Leica TCS SP2 software (Leica Microsystems, Heidelberg, Germany).

Anti-TfR and Tf Uptake Assays—Transfected or infected cells grown on coverslips were stained in serum-free medium (containing 0.1% BSA, 10 mg/ml HEPEs) for 2 h at 37 °C. Cells were then incubated at 37 °C for 30 min in the same medium containing either 10 μg/ml Tf-Alexa-488, -546 or -594, 3 μg/ml EGF-TxR, or 20 μg/ml anti-CD71. Intensity of fluorescence per pixel was determined on the whole fluorescent secondary Ab. Cells were washed, fixed, and permeabilized as previously. When indicated, CD8 staining was performed as described above, and Nef staining was performed using the goat anti-Nef polyclonal antibody and a Cy5-conjugated anti-IgG as secondary antibody. The cells were then analyzed by confocal microscopy. Tf uptake was quantified at the single-cell level by confocal laser scanning microscopy using Bio-Rad MRC1000 software. Double immunofluorescence was performed using the 488-, 546- and 594-nm laser lines. In each experiment, the laser beam and the photomultipliers were adjusted to the Alexa signal of untransfected cells to avoid saturation of the signal when Tf was measured on a 250-gray values color scale. For each sample, at least 100 cells were used and the analysis was performed on GFP-positive cells with a Coultronics Epics Elite instrument. For immunofluorescence analysis, cell surface-associated anti-TIR was revealed by incubation for 1 h at 4 °C with a Cy5-conjugated anti-IgG. After washing, cells were fixed as described above, and the analysis was performed on GFP-positive cells with a Coultronics Epics Elite instrument. For immunofluorescence analysis, cell surface-associated anti-TIR was revealed by uptake of 3 μg/ml of Tf-anti-IgG. The cells were then analyzed by confocal microscopy. In each experiment, the laser beam and the photomultipliers were adjusted to the Cy5 signal of untransfected cells in order to avoid saturation when Tf was measured on a 250-gray values color scale. For each experiment, cell surface and cellular optical slices of 50 different cells were recorded. NIH Image software was used to quantify the intensity of fluorescence (mean intensity of fluorescence per pixel) on GFP-positive cells using a 0–250-gray color scale. All data were saved in different series, and statistical analysis was done. The confidence limits of the results were assessed from Student's t test.

Cell Surface Expression of Transferin Receptor (TfR)—The surface expression of TfR was analyzed by flow cytometry or indirect immunofluorescence. Transfected cells were incubated for 1 h at 4 °C with 20 μg/ml anti-human FcγRIII (MCA160, Santa Cruz). Fixation was carried out with 1% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline (PBS). Double immunofluorescence was performed using the 488-, 546- and 594-nm laser lines. In each experiment, the laser beam and the photomultipliers were adjusted to the Cy5 signal of untransfected cells in order to avoid saturation when Tf was measured on a 250-gray values color scale. For each sample, cell surface and cellular optical slices of 50 different cells were recorded. NIH Image software was used to quantify the intensity of fluorescence (mean intensity of fluorescence per pixel) on GFP-positive cells using a 0–250-gray color scale. All data were saved in different series, and statistical analysis was done. The confidence limits of the results were assessed from Student's t test.

Flow Cytometry Analysis of Tf and TfR Internalization—24 h after transfection with CD8-Nef expression vectors, cells were serum-starved for 1 h at 37 °C, transferred on ice, washed in cold PBS, and labeled at 4 °C for 1 h either with 10 μg/ml Tf-Alexa-488 in the assay medium (RPMI containing 0.2% BSA, 10 mM HEPEs) or with a FITC-conjugated anti-TIR in PBS-BSA 0.2%. Cells were then washed twice with the assay medium, and an aliquot was transferred in cold PBS. The remaining cells were transferred in the same medium at 37 °C to allow internalization of Tf-Alexa-488 or FITC-anti-TIR for different periods of time. Each sample was divided into two aliquots. One aliquot was washed for 2 min in cold assay media adjusted to pH 4 (acid wash) to remove the surface-bound Tf-Alexa-488 or the remaining FITC-anti-TIR. The other aliquot was used to quantify the total cell-associated TF-Alexa-488 or FITC-anti-TIR. After washing, the cells were assayed for cell surface expression of the CD8 chimera with an ECD-conjugated anti-CD8 in PBS containing 0.2% BSA for 1 h at 4 °C. The cell-associated anti-CD8 fluorescence was then analyzed by flow cytometry on CD8-positive cells. The percentage of internalized Tf was calculated as follows:

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\text{m} = \frac{m_X - m_T}{m_T} 
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where \( m_X \) is the total mean fluorescence obtained at time zero, \( m_T \) is the mean fluorescence obtained at time zero after internalization, and \( m_T \) is the mean fluorescence obtained at time zero after acid wash. When indicated, CD8 staining was performed as described above, and Nef staining was performed using the goat anti-Nef polyclonal antibody and a Cy5-conjugated anti-IgG as secondary antibody. The cells were then analyzed by confocal microscopy. Tf uptake was quantified at the single-cell level by confocal laser scanning microscopy using Bio-Rad MRC1000 software. Double immunofluorescence was performed using the 488-, 546- and 594-nm laser lines. In each experiment, the laser beam and the photomultipliers were adjusted to the Cy5 signal of untransfected cells in order to avoid saturation when Tf was measured on a 250-gray values color scale. For each sample, cell surface and cellular optical slices of 50 different cells were recorded. NIH Image software was used to quantify the intensity of fluorescence (mean intensity of fluorescence per pixel) on GFP-positive cells using a 0–250-gray color scale. All data were saved in different series, and statistical analysis was done. The confidence limits of the results were assessed from Student's t test.
FIG. 1. **Nef alters the distribution of early endosomal markers.** *A*, HeLa cells expressing CD8-Nef were stained by indirect immunofluorescence for EEA1 and TfR (red) and by direct immunofluorescence for CD8-Nef (green) 24 h after transfection. *B*, HeLa cells were transfected with the CD8-Nef expression vector in combination with vector expressing GFP-Rab5, GFP-Rab4, or GFP-Rab7. 24 h later, the cells were stained by indirect immunofluorescence for CD8-Nef (red). Staining was visualized by confocal microscopy. Insets show higher magnification of representative areas. Areas of co-localization are seen in yellow (arrows) in the merged images. Scale bar, 10 μm.
incubated for 10 min with 10 μg/ml Tf-Alexa-488 in the assay medium (RPMI containing 0.2% BSA, 10 mM HEPES) to allow continuous internalization of Tf. Surface-bound Tf was removed by washing for 2 min with the assay medium adjusted to pH 4 (acid wash) followed by washes with cold PBS containing 1 mg/ml of unlabeled holo-Tf. An aliquot was transferred in cold PBS to quantify the cell-associated Tf-Alexa-488 at time zero. The remaining cells were transferred in the assay medium containing 1 mg/ml unlabeled holo-Tf and incubated at 37 °C for different periods of time to allow Tf-Alexa-488 to recycle back to the cell surface. After washing, the cells were then washed with the same cold medium, followed by washes in cold PBS, and assayed for cell surface expression of the CD8 chimera as indicated above. The cell-associated Tf-Alexa-488 was quantified by flow cytometry on CD8-positive cells. The percentage of Tf recycling was calculated as follows, \( \frac{m_X}{m_0} \times 100 \). 

\( m_X \) is the mean fluorescence obtained at each time point, and \( m_0 \) is the mean fluorescence obtained at time zero.

Western Blot Analysis—Expression of wild-type and mutated Nef-GFP fusions, native non-tagged Nef proteins, and CD8-Nef chimeras was analyzed by Western blotting, as described previously (17, 23), from transfected or infected cells using anti-GFP, anti-Nef, and anti-CD8 antibodies, respectively.

RESULTS

HIV-1 Nef Is Associated Mainly with Early/Sorting Endosomes and Affects the Distribution of Early Endosomal Markers—

Previous studies have shown that HIV-1 Nef is expressed mainly in a perinuclear endosomal compartment where it stabilizes the attachment of AP-1 and AP-3 complexes to membranes by an ARF1-independent mechanism (17, 18). To explore whether this Nef property underlies a widespread alteration of the trafficking of membrane proteins within the endosomal compartments, we first analyzed the distribution of Nef in relation to different endosomal markers in HeLa cells expressing a CD8-Nef chimera containing Nef as a cytoplasmic domain. This chimera retains the full activity of the native myristoylated Nef and thus represents a valuable tool for analyzing the interaction between Nef and the endocytic machinery (17, 18, 20, 24–27). As evidenced in Fig. 1A, the distribution of early endosomal markers, such as early endosomal antigen 1 (EEA1) and TIR, was modified dramatically by Nef expression. Both the TIR and EEA1-positive vesicles became concentrated in the perinuclear region of cells expressing CD8-Nef as compared with the more diffuse vesicular staining observed in untransfected cells.
FIG. 3. **Nef modulates the cell surface expression of TIR.** A, immunofluorescence analysis of cell surface TIR. HeLa cells expressing Nef-GFP (upper panels) or GFP (lower panels) were stained at 4 °C with anti-TIR followed by Cy5-conjugated anti-IgG. The cells were analyzed by confocal microscopy. Nef-GFP was visualized by direct fluorescence (green, left panel), and for each sample TIR expression (red) was recorded from cell surface (middle panels) and medial (right panels) optical slices. The asterisks correspond to transfected cells. Scale bar, 10 μm.

B

C

**FIG. 3.** **Nef modulates the cell surface expression of TIR.** A, immunofluorescence analysis of cell surface TIR. HeLa cells expressing Nef-GFP (upper panels) or GFP (lower panels) were stained at 4 °C with anti-TIR followed by Cy5-conjugated anti-IgG. The cells were analyzed by confocal microscopy. Nef-GFP was visualized by direct fluorescence (green, left panel), and for each sample TIR expression (red) was recorded from cell surface (middle panels) and medial (right panels) optical slices. The asterisks correspond to transfected cells. Scale bar, 10 μm.
involved in the regulation of vesicular trafficking within the endosomal pathway: Rab5, Rab4, and Rab7. The function of these GTPases is restricted to the membrane compartments where they are localized; the early/sorting endosomes are enriched in Rab5 and Rab4, whereas the late endosomes instead contain Rab7 (28). As shown in Fig. 1B, CD8-Nef co-localized extensively with Rab5- and Rab4-positive structures. Indeed, the GFP-Rab4 staining actually surrounded most of the CD8-Nef positive vesicles. Conversely, little co-localization was detectable with GFP-Rab7. These results extend our previous data indicating that Nef is associated mainly with the early endosomal compartment (18) but also show that Nef alters the distribution of early endosomal markers.

**Nef Alters Endosomal Trafficking of TfR but Not Epidermal Growth Factor Receptor (EGFR)—**According to the results reported in Fig. 1A, we investigated whether the expression of Nef affects the trafficking of TfR, for which endocytosis and recycling have been extensively characterized (29). HeLa cells were transfected with a vector expressing a Nef-GFP fusion, and antibody uptake experiments were performed 24 h later. Cells were incubated with anti-TfR for 30 min at 37 °C and then fixed and analyzed by indirect immunofluorescence (Fig. 2A, upper panels). In untransfected cells, numerous punctuate endosomal structures were labeled with the anti-TfR antibody. In contrast, a weak intracellular anti-TfR staining was observed in Nef-expressing cells, indicating that Nef dramatically impairs the uptake of anti-TfR.

Similarly, an alteration of TfR trafficking was also observed by using Alexa-594-conjugated Tf (Tf-Alexa-594) in an uptake experiment. 24 h after transfection, cells were depleted of endogenous Tf and then allowed to continuously internalize Tf-Alexa-594 for 30 min at 37 °C before fixation (Fig. 2B); expression of Nef-GFP (upper panels) dramatically reduced intracellular Tf staining compared with untransfected and control cells expressing GFP (lower panels). Quantitative analysis from the fluorescence images showed that cells expressing Nef-GFP, or the CD8-Nef chimera, exhibited a decrease of about 60–70% of Tf uptake compared with control cells (Fig. 2C). In contrast, a non-myristoylated form of Nef (NefG2A-GFP) failed to disturb Tf uptake, showing that the association of Nef on membranes is, as for other known Nef functions (for review, see Refs. 4 and 5), strictly required for its effects on TfR trafficking.

We further explored whether Nef could alter the trafficking of the epidermal growth factor receptor. EGF-induced EGFR activation promotes acceleration of receptor internalization and retention of the EGF-EGFR complexes into early/sorting endosomes before degradation into the lysosomal compartment, even if a weak recycling of the EGFR bound to EGF can be observed (30, 31) (for review, see Ref. 32). HeLa cells were transfected with the Nef-GFP expression vector, and EGFR uptake was analyzed 24 h later by incubation with EGF-TxR at 37 °C for 30 min before fixation. As shown in Fig. 2A (lower panels), Nef did not seem to alter the trafficking of EGFR, because no difference in the intracellular accumulation of EGF-TxR was observed between Nef-expressing cells and untransfected cells.

Together these data show that Nef specifically impairs the endosomal trafficking of a recycling membrane receptor such as TfR, whereas the trafficking of a receptor that is mainly addressed to the degradation compartments, such as the EGFR, is preserved.

**Nef Modulates the Cell Surface Expression of TfR—**Because our data showed that Nef altered the endosomal trafficking of TfR and induced its accumulation within a perinuclear endosomal compartment (see Fig. 1A), we next analyzed whether Nef affected the cell surface level of TfR at steady state. HeLa cells were transfected with either the Nef-GFP or GFP expression vector, and the cell surface expression of TfR was assessed by immunofluorescence on non-permeabilized cells (Fig. 3A). Compared with untransfected cells or control cells expressing GFP (Fig. 3A, lower panels), Nef-GFP expressing cells exhibited a significant decrease in their surface level of TfR (upper panels). Indeed, quantitative analysis from the fluorescence images showed that cells expressing Nef-GFP exhibited a decrease of about 50–60% of TfR surface levels compared with control cells or neighboring untransfected cells (Fig. 3B). Such an effect of Nef on the cell surface expression of TfR was then confirmed by flow cytometry analysis (Fig. 3C). These data show that Nef expression results in a significant decrease of the level of TfR at the cell surface. Moreover, this finding explains the inhibition of anti-TfR and Tf uptake reported in Fig. 2.

**Nef Also Promotes Alterations of TfR Trafficking in T Lymphocytes—**To validate that the Nef-induced alterations of TfR trafficking could be observed also in cells relevant to HIV-1 infection, we next studied the effects of Nef expression in a T lymphocyte cell line such as CEM cells. Cells were transfected with either Nef-GFP or GFP expression plasmids, and TfR-ligand uptake experiments were first performed. 24 h later, cells were incubated with Tf-Alexa-546 for 30 min at 37 °C, as indicated above, and then fixed (Fig. 4A). As observed in HeLa cells, the expression of Nef-GFP in CEM cells (Fig. 4A, upper panels) dramatically reduced intracellular Tf staining compared with untransfected and control cells expressing GFP (lower panels). Second, we analyzed by flow cytometry whether Nef-induced alteration of Tf uptake was also related to a decrease in the steady state surface level of TfR in CEM cells (Fig. 4B). As expected, Nef-expressing CEM cells exhibited a decrease of about 50% of TfR surface levels compared with control cells. These results show that the expression of Nef causes similar effects in both lymphoid and non-lymphoid cells, indicating that the effects of Nef on endosomal trafficking are related to a widely conserved mechanism.

**Nef Specifically Perturbs Recycling of TfR Back to the Cell Surface—**After binding of its ligand, TfR is constitutively internalized from the plasma membrane via clathrin-coated vesicles, traffics through the early/sorting endosomes, and is then recycled back to the cell surface from the early/recycling endosomal compartment (29). Since the relative rates of internalization of the cell surface expression of TfR was assessed as described in A. The intensity of the fluorescence corresponding to Cy5 staining was quantified from 50 transfected (Nef-GFP- or GFP-expressing cells) or untransfected cells (Mock) as described under “Experimental Procedures.” The results are expressed as the percentages of the mean fluorescence intensity determined in transfected cells relative to that determined in untransfected cells (100%, mock). Values are the means of three independent experiments. Errors bars represent 1 S.D. from the mean. The asterisk indicates a confidence limit of >99.9% (p < 0.001) for Student’s t test in comparison with the control cells. C, flow cytometry analysis of cell surface TfR. HeLa cells expressing Nef-GFP or GFP were stained at 4 °C with anti-TfR followed by PE-conjugated anti-IgG, and flow cytometry analysis was then performed on GFP-positive cells. Representative fluorescence-activated cell sorter profiles from cells expressing Nef-GFP (black curve) or GFP (bold dotted curve) are shown (left panel). No cell surface staining was detected with the PE-conjugated anti-IgG (dotted curve). The relative cell surface expression of TfR in Nef-expressing cells was quantified (right panel). The cell surface TfR was assessed as described previously, and the results are expressed as the percentage of the mean fluorescence intensity determined in Nef-GFP expressing cells (white bar) relative to those determined in GFP control cells (100%, black bar). Values are the means of four independent experiments. Errors bars represent 1 S.D. from the mean. The asterisk indicates a confidence limit of >99.9% (p < 0.001) for Student’s t test in comparison with control cells.
FIG. 4. Nef also promotes alterations in TfR trafficking in T lymphocytes. A, CEM T cells expressing Nef-GFP (upper panels) or GFP (lower panels) were serum-starved and then incubated with Tf-Alexa-546 for 15 min at 37 °C before fixation. Staining was visualized by fluorescence microscopy. Merged images show both GFP and Tf-Alexa-546 (red) labeling. The asterisks correspond to transfected cells. Scale bar, 15 μm. B, flow cytometry analysis of cell surface TfR. CEM cells expressing Nef-GFP or GFP were stained at 4 °C with anti-TfR followed by PE-conjugated anti-IgG, and flow cytometry analysis was then performed on GFP-positive cells. Representative fluorescence-activated cell sorter profiles from cells expressing Nef-GFP (black curve) or GFP (bold dotted curve) are shown (upper panel). No cell surface staining was detected with the PE-conjugated anti-IgG (dotted curve). The relative cell surface expression of TfR in Nef-expressing cells was quantified (lower panel). The cell surface TfR was assessed as described previously, and the results are expressed as the percentage of the mean fluorescence intensity determined in Nef-GFP-expressing cells (white bar) relative to those determined in GFP control cells (100%, black bar). Values are the means of four independent experiments. Error bars represent 1 S.D. from the mean. The asterisk indicates a confidence limit of >99.9% (p < 0.001) for Student’s t test in comparison with control cells.
ization and recycling determine the steady state expression of TfR at the cell surface, the accumulation of TfR in endosomal structures observed in Nef-expressing cells could be related to a stimulation of internalization and/or an inhibition of recycling. We thus explored the potential impact of Nef on the kinetics of internalization and recycling of TfR. The kinetic of Tf internalization was first analyzed in cells expressing the CD8-Nef chimera. 24 h after transfection, the cells were first depleted of endogenous Tf, and the cell surface TfR was loaded with Tf-Alexa-488 at 4 °C for 1 h. The cells were then incubated at 37 °C for various periods of time, and the cell-associated fluorescence was measured on CD8-positive cells by flow cytometry as indicated under “Experimental Procedures.” As shown in Fig. 5A, the rate of Tf internalization was not significantly different in cells expressing CD8-Nef compared with control cells. This finding indicates that Nef does not affect clathrin-mediated internalization of TfR from the cell surface.

We next analyzed whether Nef affected the recycling of TfR back to the plasma membrane. As before, cells were first depleted of endogenous Tf and then incubated for 10 min at 37 °C with Tf-Alexa-488 to allow continuous internalization. The surface-bound Tf-Alexa-488 was removed by acid wash, and the cells were then incubated again at 37 °C for different periods of time to allow the recycling of Tf and its subsequent release in the extracellular milieu. The remaining cell-associated fluorescence was finally measured on CD8-positive cells by flow cytometry, and the rate of Tf recycling was calculated as indicated under “Experimental Procedures.” As shown in Fig. 5B, Nef expression drastically reduced the rate of Tf recycling to the cell surface. These results indicate that Nef specifically impairs the recycling of internalized TfR back to the cell surface. This effect likely explains the intracellular accumulation of TfR in endosomal compartments at steady state and the low cell surface expression of TfR observed in Nef-expressing cells (see Figs. 1 and 3, respectively).

Nef Inhibits Formation of the Tubular Recycling Compartment—Internalized TfR can be recycled back to the plasma membrane directly from the early/sorting endosomes or after delivery to a specialized endosomal recycling compartment (ERC) (33–36). Therefore, the mechanism for Nef effects on TfR recycling could be related to a specific impairment of the ERC. To test this hypothesis, we studied the distribution of ERC-specific markers, such as the Rab11 GTPase and the Rme1 protein, in cells expressing Nef. Both Rab11 and Rme1 have been implicated in the modulation of protein transport through the ERC (35, 37). HeLa cells were transfected with vectors expressing either GFP-Rab11 or GFP-Rme1 alone or in combination with the CD8-Nef expression vector and were then analyzed by confocal microscopy 24 h later. As shown in Fig. 6A, Nef altered the punctate vesicular expression pattern of GFP-Rab11, leading to a strong perinuclear concentration of enlarged GFP-Rab11-positive structures in which the CD8-Nef chimera totally co-localized. This Nef-induced redistribution of GFP-Rab11 was confirmed by quantitative analysis of the staining patterns in the peripheral and perinuclear regions (Fig. 6C). In addition, Nef also severely altered the staining pattern of GFP-Rme1 (Fig. 6B). As reported previously (35), GFP-Rme1 was associated mainly with membrane structures that extend from the perinuclear region to the cell periphery when expressed alone (Fig. 6B, left panel). In co-transfected cells, Nef co-localized extensively with Rme1, but the GFP-Rme1 staining on tubular structures was collapsed to small “sticks” and vesicles (Fig. 6B, right panels). As shown in Fig. 6D, the length of the GFP-Rme1-positive tubular structures was significantly reduced in Nef-expressing cells (4.1 ± 1.2 μm in cells expressing GFP-Rme1 alone versus 1.3 ± 0.3 μm in cells co-expressing GFP-Rme1 and CD8-Nef). Altogether, these findings indicate that the expression of Nef results in a severe morphological as well as functional impairment of the sorting/recycling endosomal compartment.

The Leucine-based Motif of Nef Is Required for Alteration of TfR Recycling—Nef-mediated alterations of trafficking of receptors such as CD4, CD28, and DC-SIGN are related to the C-terminal leucine-based motif of Nef required for direct interaction with AP complexes (9, 11, 18, 20, 25, 26). In contrast, Nef-induced down-modulation of MHC-I is determined by dis-
tinct motifs located in the N-terminal part of Nef: an acidic cluster (62EEEE65), and the Src homology 3-binding motif (72PXXP75) (21, 27, 38). To analyze the contribution of these motifs to the Nef-induced alteration of TfR trafficking, alanine substitutions were introduced in Nef to generate the Nef-LL164–5/AA, Nef-E62EEE/4A, and Nef-PXXP/A-GFP mutants. HeLa cells were transfected with plasmids encoding the WT or mutated Nef-GFP proteins, and Tf uptake was assessed (Fig. 7A). The analysis showed that Tf uptake was similarly altered in cells expressing WT Nef or NefE62EEE/4A and Nef-PXXP/A (not shown) mutants. Conversely, Tf staining in cells expressing the NefLL164–5/AA mutant was not different from that observed in untransfected cells, even though this mutant was expressed efficiently (Fig. 7D, upper panel). Similar results were obtained in HeLa cells transfected with an expression plasmid (Fig. 7B) that encodes a non-tagged myristoylated HIV-1 Nef protein (Fig. 7D, middle panel). In addition, the NefLL164–5/AA mutant failed to modulate the cell surface expression of Tf at steady state (not shown). These data indicate that the Nef leucine-based motif is crucial for Nef-induced alteration of Tf trafficking, and the motifs required for down-regulation of MHC-I are dispensable.

The role of the leucine-based motif in TfR recycling was then addressed directly. Although well expressed (Fig. 7D, lower panel), CD8-NefLL164–5/AA failed to alter the recycling rate of Tf (Fig. 7C). Again, neither the WT CD8-Nef chimera nor the LL164–5/AA mutant affected the internalization of Tf (Fig. 7C, inset). These results indicate that the integrity of the Nef leucine-based motif is required for the inhibition of Tf recycling. Since it has been reported that the integrity of the
leucine-based motif is crucial for the association of Nef with both AP-1 and AP-3 complexes (18, 19), these results suggest that the Nef-induced alteration of the recycling compartment is likely related to the recruitment and stabilization of these complexes on endosomal membranes.

The Effect of Nef on TfR Trafficking in HIV-1-infected Cells Correlates with Nef-mediated Enhancement of Viral Infectivity—The Nef effect on TfR trafficking was assayed in cells infected with HIV-1 to confirm that this activity was also observed during viral replication. HeLa-CD4 cells were infected with either wild type, NefLL164–5/AA-mutated, or Nef-deleted HIV-1 strains and were then subjected to Tf-Alexa-594 uptake as described above 24 h after infection. Infected cells were identified by immunofluorescence with an antibody to the viral capsid protein (p24). As in cells transfected with Nef-expression plasmids (see Fig. 2), a significant decrease of endocytic structures stained with Tf-Alexa-594 was apparent in cells infected with WT viruses (Fig. 8A, left panels) but not in cells infected with either NefLL164–5/AA mutant or Nef-deleted viruses that showed a Tf staining similar to uninfected cells (Fig. 8A, middle and right panels). Quantitative analysis from the fluorescence images showed that most of the cells infected with the WT viruses exhibited a decrease of about 40–50% of Tf-Alexa-594 intracellular staining compared with cells infected with the NefLL164–5/AA mutant or Nef-deleted viruses (Fig. 8B). Similar expression levels of Nef proteins were found in cells infected with the WT or the NefLL164–5/AA mutant viruses (Fig. 8C) and were equivalent to those observed in HeLa cells transfected with the different Nef expression plasmids employed throughout our study (compare Figs. 7D and 8C). These results indicate that Nef also alters the trafficking of TfR when it is expressed during virus replication.

Finally, we investigated the relationship between the Nef-induced alterations of endosomal trafficking and the Nef-mediated enhancement of viral infectivity by analyzing the effect of the LL164–5/AA mutation on the infectivity of HIV-1. Virions produced by transient transfection of CD4-negative 293T cells were used to measure infectivity, both at 24 and 48 h after infection, in an infectious center assay in which HeLa-CD4 cells were used as targets (22). The results showed that both NefLL164–5/AA mutant and Nef-negative viruses were similarly less infectious than the wild type (Fig. 8D), indicating that the majority of the effect of Nef on viral infectivity depends on its leucine-based motif. Moreover, this leucine-dependent effect on viral infectivity could not be attributed to Nef-induced down-regulation of CD4, because the virions were produced from CD4-negative cells.

Altogether, the results gathered from Figs. 7 and 8 establish a correlation between Nef-induced membrane stabilization of
FIG. 8. Nef-induced alterations on TfR trafficking correlate with its requirement for the full infectivity of HIV-1 virions. A, inhibition of Tf uptake in HIV-1-infected cells. HeLa-CD4 cells were infected with the wild type, NefLL164–5/A mutant, or Nef-deleted (ΔNef) NL4-3 HIV-1 strains. 24 h postinfection, the cells were serum-starved and incubated for 30 min with Tf-Alexa-594 at 37 °C as indicated in Fig. 2B. The cells were then fixed, permeabilized, and stained by indirect immunofluorescence with anti-p24 (upper panels) to identify infected cells. Cells

B

C

D

The fluorescence intensity was measured as described in Fig. 2B. The results are expressed as the mean ± SEM of three independent experiments.

The significance of the differences between groups was determined by one-way ANOVA followed by Tukey's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the wild type.

The effect of Nef on HIV-1 infectivity was also determined by measuring the number of blue cells per field (blue cells/hrp24) in the presence of various concentrations of Nef. The results are shown in Fig. 3D. The significance of the differences between groups was determined by one-way ANOVA followed by Tukey's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the wild type.

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AP complexes, Nef-induced effects on the early/recycling compartment, and Nef-mediated enhancement of viral infectivity.

DISCUSSION

We have demonstrated here that HIV-1 Nef dramatically affects the morphology and function of the endocytic recycling compartment, leading to alterations of the trafficking of Tir, a membrane receptor that recycles to the plasma membrane through sorting/recycling endosomes. By contrast, Nef does not affect the trafficking of EGF-R, a receptor that is rather addressed to the late endosomal compartments after internalization (32). Like Nef-induced modulation of the cell surface expression of CD4 (22), these effects require the integrity of the leucine-based motif located in the C-terminal loop of Nef. Moreover, we confirm that the Nef leucine-based motif is crucial for Nef-mediated enhancement of HIV-1 infectivity even when virions are produced from CD4-negative cells. Since the leucine-based motif constitutes the critical determinant of Nef required for interaction with the AP-1 and AP-3 complexes and for their stabilization on membranes (18–20, 22, 25), our results suggest that the requirement of Nef for the full infectivity of HIV-1 virions is related to a general alteration of the sorting/recycling compartments in virus producing cells.

In agreement with the Nef-induced changes in the morphology of the endosomal compartments previously observed by electron microscopy (17, 39, 40), Nef extensively co-localized with early endosomal markers, such as EEA1, Rab4 and Rab5, and provoked a striking redistribution of markers of the early/recycling endosomal compartment, such as Tir, Rab11, and also Rme1, a protein that regulates the function of the recycling compartment (35). This redistribution was characterized by a strong concentration of Tir- and Rab11-containing vesicles in a perinuclear compartment and an evident collapse of the Rme1-positive tubulovesicular structures. These observations suggest that Nef acts mainly at the level of early/recycling endosomes to induce severe morphological alterations of the recycling compartment. The sorting endosomal compartment is indeed a critical platform of the endocytic pathway where vesicles from both the plasma membrane and the TGN deliver their cargos. There, proteins destined for the degradation compartments, such as EGF-R, segregate from those that are recycled to the plasma membrane or the TGN, such as Tir (30, 41), even if a weak recycling of the EGF-R bound to EGF can be observed (32).

Functionally, the Nef-induced alterations of the recycling compartment result in an inhibition of the recycling of Tir back to the cell surface. This observation confirms that Nef has a widespread effect on the trafficking of membrane proteins within the endocytic pathway. In addition to CD4, MHC-I, MHC-II, CD28, and DC-SIGN (6, 7, 9–11), the Tf-R can now be included on the list of cellular proteins in which trafficking is affected by Nef. In the case of Tir, Nef-mediated inhibition of recycling results in a net decrease of the expression of this receptor at the plasma membrane and induces its accumulation in a perinuclear endosomal compartment. Since Tir is a model for studying clathrin-mediated receptor endocytosis and recycling, our results anticipate that Nef should affect other proteins that use this recycling pathway. Notably, recycling of Tir to the plasma membrane can occur directly from the early sorting endosomes or through the perinuclear recycling compartment (29, 33, 36), and further analyses are needed to determine whether Nef acts on both pathways or specifically alters the perinuclear recycling pathway.

Interestingly, the effects of Nef on Tir cycle are reminiscent of those induced by expression of the dominant negative Rme1 (G429R) mutant (35). Nef induces an intracellular accumulation of the receptor as a result of a reduced rate of recycling, whereas the internalization of Tir is not significantly affected. In addition, both Nef and the Rme1 mutant disturb the retrograde transport from early/sorting endosomes to the TGN without affecting transport to late endosomes and lysosomes (35, 42). As proposed to explain the inhibitory activity of the Rme1 mutant, Nef may behave as an inhibitor of the formation and/or maturation of transport vesicles and tubules throughout the early/sorting endosomal compartment. This suggests that Nef acts primarily on sorting endosomes and thus affects the morphology and function of the recycling compartment.

How might the known properties of Nef cause such a disturbance? The recycling compartment derives, at least in part, from vesicles originated at the level of the early/sorting endosomes (for review, see Ref. 41). The formation and budding of endosomal vesicles require the association of coat components with membranes, but their release is a prerequisite for subsequent membrane fusion. The underlying molecular mechanism for the inhibitory effect of Nef may relate to its ability to stabilize the association of AP-1 and AP-3-containing coats on endosomal membranes (18). Indeed, Nef mimics a constitutively active form of ARF1 and is able to dock these AP complexes via its leucine motif (19), causing their persistent attachment to endosomal membranes. Accordingly, Nef may promote an accumulation of nascent coated vesicles at the level of sorting endosomes, resulting in a large expansion of this endosomal compartment in which CD4, Tir, and possibly other proteins are sequestered (18). Furthermore, this could explain the apparent disappearance or collapse of the peripheral Rab11 recycling compartment evidenced by the shortening of the Rme1-positive tubulovesicular structures.

The Nef-mediated membrane stabilization of AP-1 and AP-3 is likely the basis for a general perturbation of endosomal trafficking in Nef-expressing cells (18). The data herein further suggest a role for AP-1 and AP-3 in the regulation of vesicular transport throughout the early endosomal compartments. AP-1 and AP-3 were originally reported to mediate the transport of a subset of proteins from the TGN to endosomes and lysosomes (43). However, it has become apparent that they are also involved in vesicular transport within the endocytic/recycling pathway. First, AP-1 has been shown to be required for retro-
grade transport from early/sorting endosomes to the TGN (44, 45). Second, AP-3 has been found to be associated at least in part with a TIR-positive compartment (46). Third, clathrin coats have been detected on vesicles budding from the early endosomal membranes (33). Interestingly, Tf recycling was inhibited by the fungal metabolite brefeldin A, an inhibitor of ARF1-dependent recruitment of coat proteins such as AP-1 and AP-3 (47–49). Nevertheless, the precise role of the AP-1 and AP-3 complexes within the recycling pathway remains to be defined, and Nef may constitute a powerful tool for this purpose.

Finally, what is the role of the perturbations of membrane protein trafficking induced by Nef in the HIV-1 life cycle? Interestingly, there is a striking correlation between the Nef-mediated enhancement of viral infectivity, the stabilization of AP-1 and AP-3 complexes on endosomal membranes, and the Nef-induced alterations of trafficking within the early/recycling endosomal compartment. All of these properties require the leucine-based motif of Nef. Here, we not only have confirmed the crucial role of the leucine-based motif of Nef for optimal viral infectivity (22) but have further emphasized that these effects are not a direct consequence of the down-regulation of CD4. It is noteworthy that these effects are apparent even when virus particles are produced from CD4-negative cells (see Fig. 5D). Although the mechanism of Nef-mediated enhancement of infectivity remains ill-defined, these observations indicate that the virological effects of Nef are related to its morphologic and functional effects on the early/recycling compartment. The precise basis of this connection between the trafficking and the virologic effects of Nef is open to question.

The mechanism(s) of Nef-mediated enhancement of infectivity remains ill-defined, these observations indicate that the virological effects of Nef are related to its morphologic and functional effects on the early/recycling compartment. The precise basis of this connection between the trafficking and the virologic effects of Nef is open to question.
Nef-induced Alteration of the Early/Recycling Endosomal Compartment Correlates with Enhancement of HIV-1 Infectivity

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