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Detection of Human Immunodeficiency Virus Type 1 Nef and CD4 Physical Interaction in Living Human Cells by Using Bioluminescence Resonance Energy Transfer

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CD4 down-regulation by human immunodeficiency virus type 1 (HIV-1) Nef protein is a key function for virus virulence. This activity may be mediated by a direct Nef-CD4 interaction. We investigated the formation, in situ, of such a complex between proteins using bioluminescence resonance energy transfer technology and coimmunoprecipitations. Our data clearly demonstrate that Nef and CD4 interact in intact human cells. Moreover, our results clearly indicate that the dileucine motif of the CD4 cytoplasmic domain, critical for the Nef-induced CD4 down-regulation, is not implicated in the Nef/CD4 complex formation in the cellular context.

The myristoylated Nef protein encoded by primate immunodeficiency viruses is essential for efficient viral replication and pathogenesis in their hosts (17, 21, 28, 30, 43). This viral protein is abundantly produced at the early stages of viral replication and displays variety of functions. Nef interferes with signal transduction pathways (19, 40, 44, 47, 54); down-modulates CD4 (18, 24, 25, 38, 39), major histocompatibility complex class I (MHC-I) (35, 46), CD3 (7, 27, 53), and CD28 (6, 50) receptors; and also enhances virion infectivity (13, 15, 45, 56) and replication capacity (2, 15, 37, 49). Several steps of the Nef-mediated CD4 down-regulation molecular mechanism are understood, like the recruitment of the μ2 chain of the adapters (11, 16, 20, 22, 32, 34, 38) and the coatomers (8, 26, 39) to redirect membrane CD4 to the lysosomal degradation pathway. However, the implication of a Nef/CD4 complex remains putative. This complex was sensed with the yeast two-hybrid system (42) and detected in vitro using truncated proteins (23). Through recent nuclear magnetic resonance (NMR) experiments (41), the dissociation constant was found to be in the submicromolar range. Furthermore, previous in vitro works (1, 41, 42) have revealed that the $L_{413}L_{414}$ motif of the CD4 cytoplasmic domain was critical in vitro for the CD4/Nef complex formation and the Nef-induced down-regulation. Up to today, the formation of this complex has never been revealed in situ with full-length Nef and CD4 proteins and the importance of the domains depicted in vitro have not been confirmed in vivo. To directly study in living human cells the ability of Nef to interact with the CD4 receptor, we use bioluminescence resonance energy transfer (BRET) (4, 10, 14, 36, 55) in HEK-293 cells. In this technique, the putative protein partners are respectively fused to *Renilla* luciferase (Rluc), and to enhanced yellow fluorescent protein (EYFP). In the presence of the substrate coelenterazine and upon its degradation by Rluc, the luminescence is transferred from Rluc to EYFP, with an efficiency depending on the proximity (<5 nm) of the fusion proteins. Protein-protein interactions are then highlighted through EYFP fluorescence emission. BRET is applicable to living cells. In order to verify the impact of the $L_{413}L_{414}$ motif of CD4 on the possible Nef-CD4 association, we also studied the CD4 $L_{413}L_{414}$-$A_{413}A_{414}$ mutant (CD4 414AA). With this technique, we demonstrate that Nef and CD4 interact in human cells. Surprisingly, the CD4 414AA mutant, which was presumed not to interact with Nef, is still recognized by Nef. To confirm these results, we perform co-immunoprecipitation assays in HEK-293 cells, with full-length native proteins, done after stabilization of the complex with a cross-linking reagent. These experiments reveal a direct interaction between Nef and CD4. Moreover we confirm that the $L_{413}L_{414}$ motif of CD4 is not implicated in the formation of this interaction. Finally, we perform BRET assays with nontagged CD4 or CD4 414AA as competitors of the fluorescent receptors. The two molecules are able to abolish the energy transfer between Nef-Rluc and CD4-EYFP or CD4 414AA-EYFP, revealing the specificity of the interactions we have observed.

The BRET and coimmunoprecitation assays were performed in HEK-293 cells (ATCC CRL-1573) to detect the putative Nef/CD4 and Nef/CD4 414AA complexes to evaluate the influence of the dileucine motif mutation in CD4 (413A,414A) on the interaction. These embryonic kidney cells, from human lineage, can be efficiently transfected using a calcium phosphate method (51, 52); they do not express CD4. Thus, no competition between endogenous CD4 and CD4-EYFP molecules should alter the efficiency of energy transfer between the tagged Nef and CD4.

The coding sequences of NefLai (HIV Databases, accession no. K02013) and CD4 were respectively inserted into the pCi-

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Neo (Promega) and pcDNA3 (Invitrogen) expression vectors. The pCML/CD4 414AA plasmid vector allowing the expression of the mutated CD4 was a gift from Didier Trono. In a preliminary experiment, to verify if a protein-protein interaction can be detected in our model, the well-documented Nef self-association (5, 29, 31) was chosen as a positive control. First, the NefLai-Rluc fusion protein was generated. The Rluc enzyme (M1 to Q311; Clontech) was fused with a four-residue spacer (GLAT) to the carboxy terminus of the full-length NefLai protein (M1 to C206; HIV Databases, accession no. K02013). To optimize protein expression, the gene of this fusion protein was cloned into the pCi-Neo vector, under the control of the immediate-early cytomegalovirus (CMV) promoter, generating the pCi-Neo/NefLai-Rluc plasmid. Using the same strategy, the EYFP (M1 to L275; Clontech) was added to the NefLai protein (M1 to C206), with a two-methionine spacer. The *neflai-eyfp* gene was then inserted into the pCI-Neo vector to generate the pCi-Neo/NefLai-EYFP plasmid. The CD4-EYFP protein was generated by fusing the EYFP (M1 to L275) with a four-residue spacer (PVAT) to the carboxy-terminal extremity of the human CD4 receptor (M1 to R450; SwissProt, accession no. P01730) and was cloned into pcDNA3, under control of the CMV promoter (pcDNA3/CD4-EYFP). The pCi-Neo/CD4 414AA-EYFP was constructed by fusing the *cd4 414AA* gene with a two-residue (VD) spacer to the EYFP gene in the pCi-Neo plasmid.

Due to the presence of a myristol group, the Nef protein is mostly anchored at the plasma membrane, but some of it is also free in the cytoplasm. Thus, results obtained with two negative controls are reported. First, as a negative control of proteins anchored in the plasma membrane, we used the pHis/β2AdrR-EYFP plasmid (kindly provided by M. Bouvier), which encodes the β2-adrenergic receptor fused to the EYFP, with pCi-Neo/NefLai-Rluc. Second, as a free cytoplasmic protein control, we used a plasmid containing two independent expression cassettes coding NefLai-Rluc and EYFP, under control of the CMV immediate-early promoter. For this, the CD4 coding sequence was eliminated from the pcDNA3/CD4-EYFP, and then the resulting expression cassette encompassing the CMV promoter and the *eyfp* was inserted into pCi-Neo/NefLai-Rluc to generate pCi-Neo/NefLai-Rluc/EYFP.

To determine in which cellular compartment the different fusion proteins are expressed in our model, we studied their localization into the HEK-293 cells. The cells (6.5×10^4) were seeded in eight-well culture slides and transfected the day after with 1 μg of the plasmid coding for the β2Adr-EYFP, Nef-Rluc, Nef-EYFP, CD4-EYFP, or CD4 414AA-EYFP fusion proteins. Two days posttransfection, the cells expressing Nef-Rluc were fixed with 1.5% paraformaldehyde and permeabilized in 1× Permwash (Pharmingen). The Nef-Rluc protein was recognized using the mouse monoclonal antibody maTG020 (kindly provided by Transgene SA) directed against Nef and labeled with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma). The cells synthesizing fluorescent proteins were only fixed. Localization of the proteins was then established upon observation of cell fluorescence with a Zeiss LSM 510 confocal microscope (Fig. 1A). The β2Adr-EYFP and the two fluorescent variants of the CD4 were essentially expressed at the plasma membrane. The Nef-Rluc and Nef-EYFP were found mainly at the plasma membrane; some patches were also detected in the cytoplasm. Thus, proteins of interest are correctly addressed in the HEK-293 cells. The supposed mechanism by which Nef affects the CD4 cell surface expression implies the recognition of the receptor by the viral protein at the plasma membrane. In order to observe if the Nef protein could colocalize with the CD4 and CD4 414AA in our model, cells were cotransfected to express simultaneously Nef or Nef-Rluc and one of the two CD4 unlabeled variants. Two days posttransfection, the HEK-293 cells were fixed and permeabilized. The CD4 receptors were labeled with an anti-CD4-PC5 antibody (Pharmingen), and Nef proteins were detected as previously described. The CD4 and CD4 414AA proteins were mostly localized at the plasma membrane; as seen by confocal microscopy (Fig. 1B) the two Nef proteins were also found principally at the cell periphery. Finally, the colocalization patterns clearly indicated that Nef and Nef-Rluc colocalize with CD4 and CD4 414AA at the plasma membrane in our expression model. Thus, a Nef-CD4 association might occur at the plasma membrane in our cells.

To verify the functionality of Nef fusion proteins, the Nef-induced CD4 down-regulation was evaluated in the HEK-293 cells. The cells (1×10^6) were cotransfected with 0.5 μg of plasmids coding for CD4 or CD4 414AA receptors and with 4.5 μg of plasmids containing the coding sequences of wild-type or fusion Nef proteins. The CD4 cell surface expression was determined 24 h after transfection by flow cytometry on cells labeled with an anti-CD4-PC5 antibody (Fig. 2). As expected, the Nef protein efficiently down-regulates wild-type CD4 (determined as reduction of CD4 cell surface expression) and has no effect on the CD4 414AA surface expression. This confirms the importance of the $L_{413}L_{414}$ motif for the CD4 down-regulation by Nef. These experiments also reveal that the fusion of Nef with EYFP or Rluc reduces the potency of Nef to down-regulate CD4 without abolishing it. Moreover, the two proteins do not acquire the ability to down-regulate the CD4 414AA mutant. Thus, these proteins can be used to perform BRET assays, in order to detect the putative Nef-CD4 interaction. To verify that the fusion of EYFP with CD4 does not alter the Nef-induced CD4 down-regulation, the presence of CD4-EYFP at the plasma membrane in Nef-expressing cells was studied by flow cytometry. Down-regulation of CD4-EYFP was not observed, either with the Nef wild type or with Nef fusion proteins (Fig. 2). The Nef-mediated CD4 internalization is a multistep process, and a lack of down-regulation may not necessary imply that the putative Nef-CD4 association does not occur. Therefore, CD4-EYFP was nevertheless used for BRET experiments to check for a direct binding of Nef with CD4.

The BRET assay was first validated in our model using the Nef-Rluc/Nef-EYFP pair. To show Nef homo-oligomerization, HEK-293 cells (1×10^7) were transfected to express the Nef-Rluc/EYFP, Nef-Rluc/β2AdrR-EYFP, or Nef-Rluc/Nef-EYFP pairs. The cells were resuspended 48 h posttransfection in HEPES-bovine serum albumin medium (51) at 10^7 cells/ml, and 10 μM of the Rluc substrate was added, (coelenterazine-f; Bioprobe). Emission spectra were measured using a spectrofluorimeter (PTI, OC-4000, MD-5020) and normalized (Fig. 3A). Fluorescence and luminescence measurements were performed at 37°C to preserve cell metabolism and plasma membrane fluidity. In the case of the control cells expressing NefLai-Rluc and EYFP or β2AdrR-EYFP, which do not in-

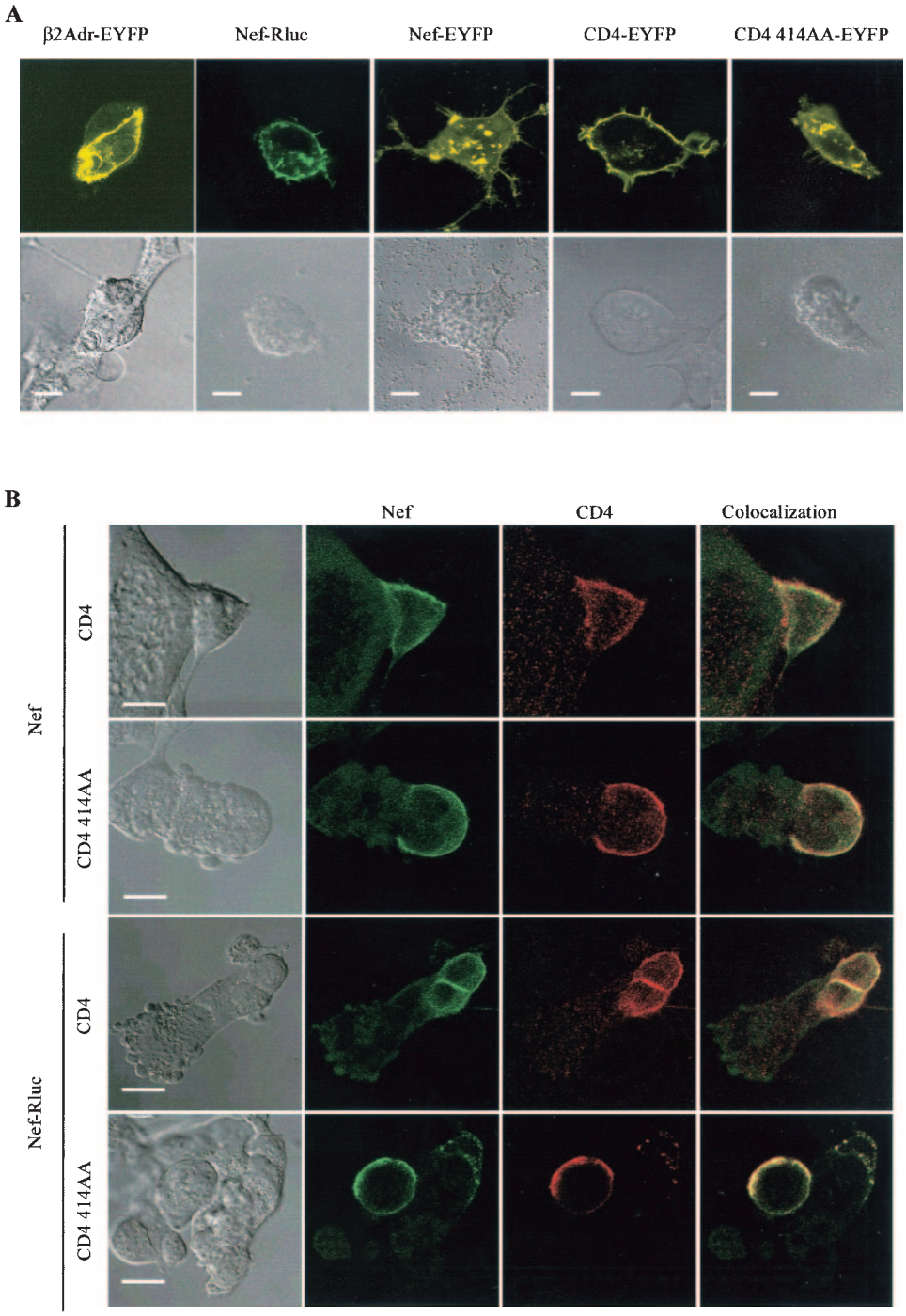

FIG. 1. The different fusion proteins are correctly addressed in HEK-293 cells. (A) HEK-293 cells (6.5×10^4) were cotransfected with 1 μg pHIS/β2Adr-EYFP, pCi-Neo/Nef-Rluc, pCi-Neo/Nef-EYFP, pcDNA3/CD4-EYFP, or pCi-Neo/CD4 414AA-EYFP. Twenty-four hours posttransfection, the cells expressing proteins fused to EYFP were fixed and observed with a Zeiss LSM 510 confocal microscope. The cells producing Nef-Rluc were fixed and permeabilized. The Nef protein was primary marked with maTG020 and labeled with an FITC-conjugated goat anti-mouse immunoglobulin G antibody. (B) To assess the colocalization of Nef or Nef-Rluc with the two CD4 variants at the plasma membrane, HEK-293 cells were cotransfected with 0.5 μg pCi-Neo/Nef-Lai or pCi-Neo/Nef-Rluc and the same amount of pcDNA3/CD4 or pCML/CD4 414AA. Forty-eight hours posttransfection, the cells were fixed and permeabilized. The Nef proteins were marked as previously. The CD4 molecules were labeled with the RPA-T4-PC5 antibody. The colocalization panels represent the overlay of CD4-PC5 and Nef-FITC fluorescences. Bars correspond to 10 μm.

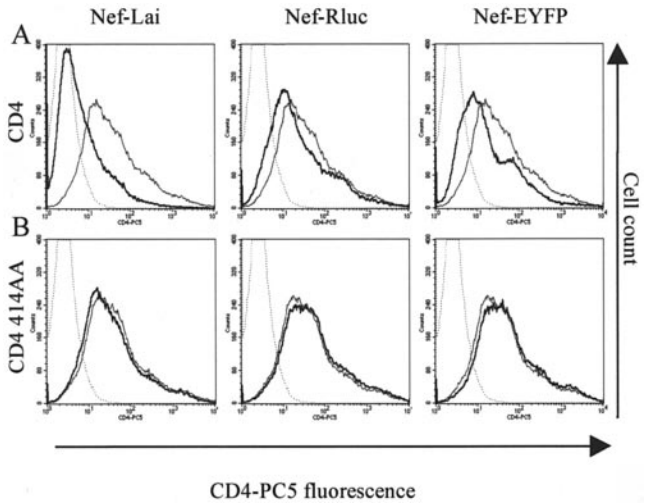

FIG. 2. The wild-type and fusion Nef proteins can induce CD4 downregulation in HEK-293 cells. (A) One million cells were cotransfected with 0.5 μg pcDNA3/CD4 and 4.5 μg pCi-Neo, pCi-Neo/NefLai, pCi-Neo/NefLai-Rluc, or pCi-Neo/NefLai-EYFP. The CD4 was labeled the day after with the RT-PA-PC5 antibody, and the cells were analyzed by flow cytometry. Abbreviations for each panel: dotted line, pcDNA3/CD4 plus pCi-Neo plus isotypic antibody; thin line, pcDNA3/CD4 plus pCi-Neo plus RPA-T4-PC5 antibody; bold line, pcDNA3/CD4 plus pCi-Neo plasmid expressing one of the Nef proteins indicated above the panels plus RPA-T4-PC5. (B) The same experiment was performed with the pCML/CD4 414AA plasmid.

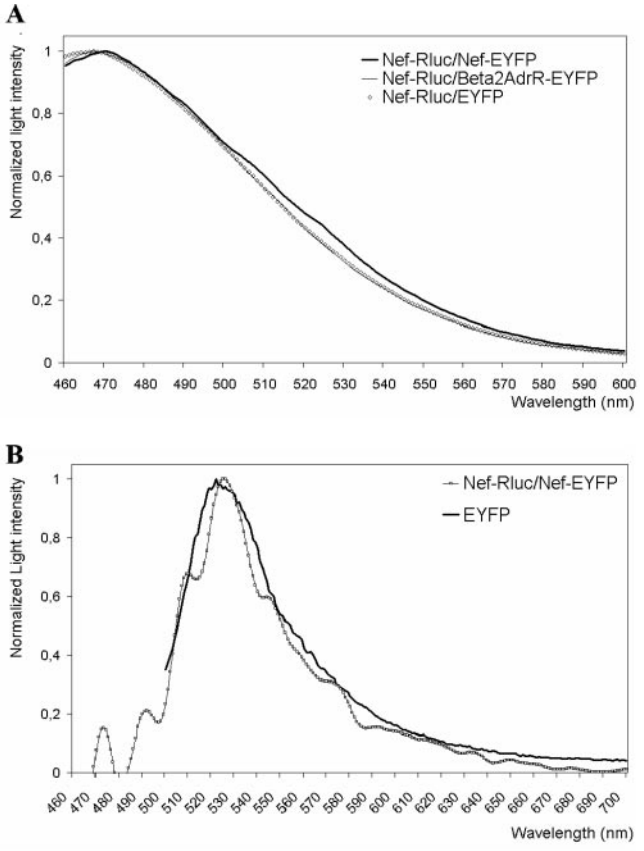

FIG. 3. The Nef dimerization can be visualized in HEK-293 cells, using the BRET technology. Ten million HEK-293 cells were transfected with 20 μg pCi-Neo/NefLai-Rluc/EYFP, 10 μg pCi-Neo/NefLai-Rluc, and 10 μg pHis/β2AdrR-EYFP or with 10 μg pCi-Neo/NefLai-Rluc and 10 μg pCi-Neo/NefLai-EYFP. (A) Two days after transfection, the signals obtained in the presence of 10 μM coelenterazine-f were detected using a PTI spectrofluorimeter. The emission spectra obtained are represented as follows: cells expressing NefLai-Rluc and EYFP, thin line; cells expressing NefLai-Rluc and β2AdrR-EYFP, dotted line; and cells expressing NefLai-Rluc and NefLai-EYFP, bold line. (B) The transfer spectrum (dotted line) is obtained by subtracting the negative control emission spectrum (NefLai-Rluc/β2AdrR-EYFP) from the spectrum obtained in cells producing NefLai-Rluc and NefLai-EYFP. This transfer signal is compared to the EYFP emission spectrum (bold line). One representative result obtained from three independent experiments is presented.

teract, only Rluc luminescence emission is expected. Indeed, no significant difference was detected between the signals of the two negative controls. In contrast, in cells producing NefLai-Rluc and NefLai-EYFP, which can oligomerize, Rluc and EYFP emission signals are expected. Then, by subtracting the β2AdrR-EYFP negative control spectrum from the one measured in cells expressing the two Nef fusion proteins, we obtain a transfer spectrum that was compared to the EYFP emission spectrum (Fig. 3B). The Nef-Rluc/Nef-EYFP transfer spectrum was indeed identical to the EYFP emission spectrum measured after excitation at 480 nm. Thus, a physical proximity of Nef-Rluc and Nef-EYFP can be detected by BRET. This highlights Nef homo-oligomerization. Our experimental system allows the detection of protein-protein interactions.

Then the association of Nef with CD4 was studied using this technique. For this, HEK-293 cells were transfected to produce the Nef-Rluc/CD4-EYFP pair and Nef-Rluc/β2Adr-EYFP as a negative control. Emission spectra were acquired as described above (Fig. 4A), and the Nef-Rluc/CD4-EYFP transfer spectrum was compared to the EYFP emission spectrum (Fig. 4B). The transfer signal corresponded to the EYFP fluorescence signal. This indicates that we detected the Nef-CD4 interaction. Furthermore, we analyzed, in the BRET assay, the binding ability of Nef-Rluc for CD4 414AA-EYFP (Fig. 4C and D). In these assays, an EYFP emission was also observed, indicating that the Nef protein can also interact with the mutated CD4 receptor.

In an attempt to visualize the Nef-CD4 and Nef-CD4 414AA associations by a second method, cells were treated with a cross-linking reagent to stabilize protein complexes before co-immunoprecipitation assays. One million HEK-293 cells were transfected with 5 μg of empty plasmid or constructions encoding Nef, CD4, or CD4 414AA proteins or cotransfected to express CD4 or CD4 414AA in the presence of Nef. Two days after transfection, the cells were metabolically radiolabeled with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (1,175 Ci/mmol) in 1 milliliter. The cell-permeant cross-linker DSP (dithiobis[succinimidylpropionate]; Pierce) was used as recommended by the manufacturer. Then the cells were lysed in phosphate-buffered saline (PBS)–0.1% sodium dodecyl sulfate (SDS)–0.3% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), pH 8. The cell lysates were centrifuged for 10 min at 2,000 *g*, cleared for 1 h with protein A/G plus agarose (Santa Cruz), and centrifuged for 10 min at 10,000 *g*. The cleared lysates were then incubated for 1 h with

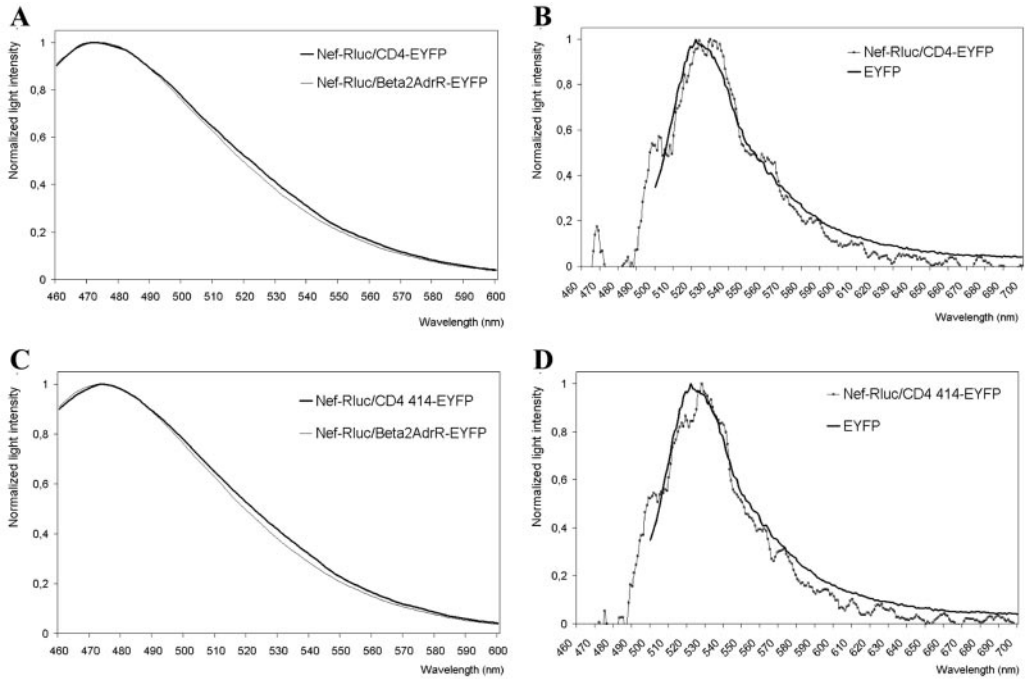

FIG. 4. The Nef/CD4 or Nef/CD4 414AA complexes can be directly detected by BRET in living HEK-293 cells. Ten million HEK-293 cells were transfected with 10 μg pCi-Neo/Nef-Rluc and 10 μg pHIS/β2AdrR-EYFP or pCi-Neo/CD4-EYFP. Twenty-four hours posttransfection, the BRET experiments were performed and the emission signals were measured (A). After subtraction of the negative control spectrum from the sample one, a distinct EYFP fluorescence emission can be observed (B). In parallel, a similar experiment was done by replacing pcDNA3/CD4-EYFP with pCi/Neo-CD4 414AA-EYFP (C and D).

maTG020 or RPA-T4 (Pharmingen) monoclonal antibodies, respectively, directed against human immunodeficiency virus type 1 (HIV-1) Nef and CD4. The immune complexes were incubated for 1 h with protein A/G plus agarose and centrifuged, and the pellets were washed six times in Tris-buffered saline –0.3% CHAPS, pH 7.4. The proteins were resolved by 12.5% SDS-acrylamide gel electrophoresis, and the gel was processed for fluorography (Fig. 5). Nef was specifically immunoprecipitated by maTG020; a background product of about 46 kDa was observed in all samples (Fig. 5A). In cells cotransfected with Nef and CD4 genes, we found that a protein with a molecular weight similar to that of CD4 coprecipitated with Nef. The same result was obtained with the CD4 414AA mutant (Fig. 5A). These data demonstrate that wild-type Nef and CD4 interact in human cells. Moreover, in this cellular context, the dileucine motif of the CD4 cytoplasmic domain, critical for the Nef-induced CD4 down-regulation, does not appear to be involved in the Nef-CD4 binding. To confirm that the proteins immunoprecipitated with Nef were effectively CD4 or CD4 414AA, immunoblotting experiments were performed. For this, 10 million HEK-293 cells were transfected to express CD4 or to produce Nef and one of the two CD4 variants. Two days later, the cells were submitted to DSP treatment and lysed, and immunoprecipitation directed against Nef or CD4 was performed. The immune complexes collected as described above were separated by electrophoresis under denaturing conditions and transferred on a polyvinylidene difluoride (PVDF) membrane (Amersham). A chemiluminescent detection of CD4 was then performed with a rabbit polyclonal antibody directed against this receptor (Santa Cruz) and an antibody against rabbit immunoglobulins coupled to horseradish peroxidase (Amersham). These experiments reveal that CD4 and CD4 414AA are specifically immunoprecipitated in the presence of Nef (Fig. 5B), indicating that the CD4 receptor and its mutant interact with Nef.

Moreover, the ability of Nef to interact with CD4-EYFP was also studied using the same procedure (Fig. 5A and B). These assays revealed that CD4 and CD4 414AA fused to the EYFP can still coimmunoprecipitate with Nef. Thus, the abrogation of the Nef-induced CD4 down-regulation observed previously with the CD4-EYFP is presumably the consequence of a steric obstruction which inhibits the recruitment of a critical protein for the down-regulation, without disrupting the Nef-CD4 interaction.

To control the specificity of the interactions observed between NefLai-Rluc and CD4-EYFP, or CD4 414AA-EYFP, competition experiments were performed using unlabeled CD4 and CD4 414AA receptors. Thus, HEK-293 cells were cotransfected to express Nef-Rluc/CD4-EYFP, Nef-Rluc/CD4 414AA-EYFP, or Nef-Rluc/β2AdrR-EYFP pairs in presence of pCi-Neo or one of the two untagged CD4 variants. Finally, the Nef-Rluc/CD4-EYFP transfer spectra, recorded in the presence or in the absence of nontagged CD4 receptors, were compared to the EYFP emission spectrum (Fig. 6A). In the absence of wild-type receptors, BRET was observed. However, in the presence of one or the other of the two competitors, the Nef-Rluc/CD4-EYFP transfer was totally abolished. The same experiments were performed with the Nef-Rluc/CD4 414AA-EYFP pair (Fig. 6B). The BRET signal previously observed with these two proteins was also abrogated by the two CD4

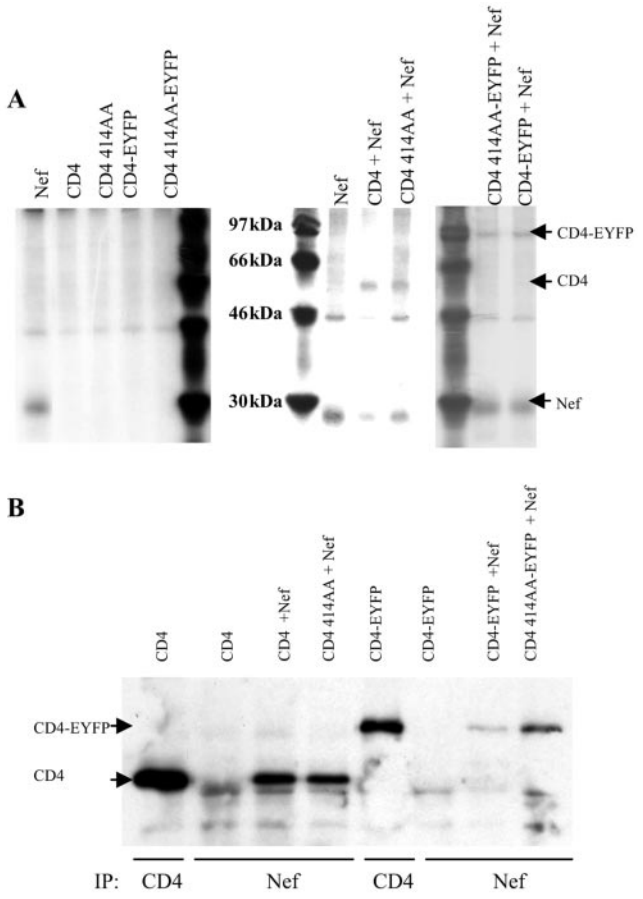

FIG. 5. Nef interacts in situ with the CD4, CD4 414AA, CD4-EYFP and CD4 414AA-EYFP receptors. (A) Proteins corresponding to CD4 or CD4-EYFP are specifically coimmunoprecipitated with the Nef antibody only in lysates of cells coexpressing Nef. One million HEK-293 cells were transfected with 5 μg pCi-Neo, pCi-Neo/NefLai, pcDNA3/CD4, pCML/CD4 414AA, pcDNA3/CD4-EYFP, or pCi-Neo/CD4 414AA-EYFP or cotransfected with 2.5 μg pCi-Neo/Nef-Lai and 2.5 μg pcDNA3/CD4, pCML/CD4 414AA, pcDNA3/CD4-EYFP, or pCi-Neo/CD4 414AA-EYFP. The next day, the cells were metabolically labeled, treated with the DSP cross-linker, and immunoprecipitated with the Nef antibody. The proteins of the immune complexes were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and revealed by fluorography. (B) The proteins coimmunoprecipitated with the Nef antibody, in the presence of Nef, are recognized by the anti CD4 antibody. Six million HEK-293 cells were transfected with 20 μg pcDNA3/CD4 or pcDNA3/CD4-EYFP or cotransfected with 10 μg pCi-Neo/Nef-Lai and 10 μg pcDNA3/CD4, pCML/CD4 414AA, pcDNA3/CD4-EYFP, or pCi-Neo/CD4 414AA-EYFP. Two days later, the cells were treated with the DSP cross-linker and lysed. The cell lysates were incubated with the Nef antibody or the CD4 antibody as indicated (IP). The proteins of the immune complexes were resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-CD4 rabbit polyclonal antibody and then with a polyclonal antibody coupled to HRP and directed against rabbit immunoglobulin G. The proteins were revealed by fluorography in the presence of the enzyme substrate.

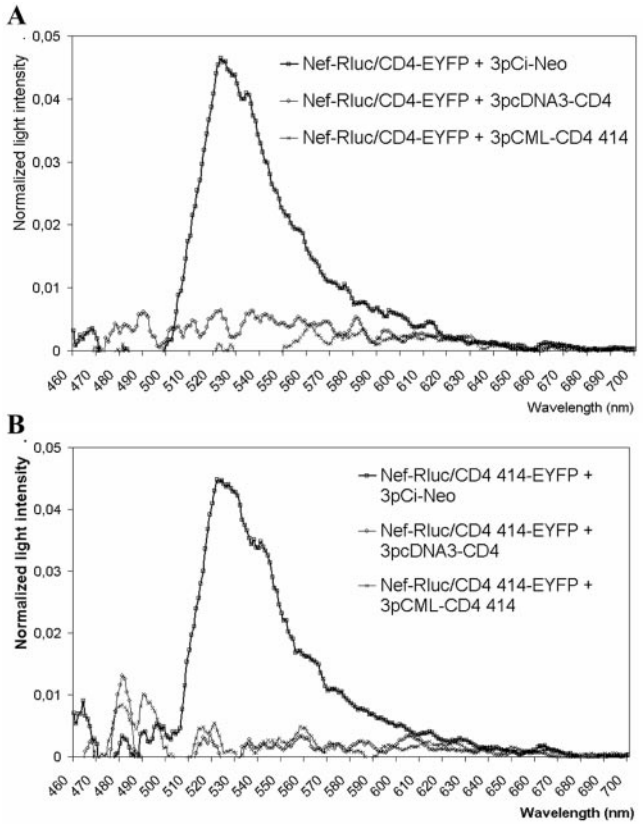

FIG. 6. The CD4 and CD4 414AA molecules can abolish Nef-Rluc/CD4-EYFP and Nef-Rluc/CD4 414AA-EYFP luminescence transfer. (A) Ten million cells were transfected with 15 μg of plasmid coding for CD4, CD4 414AA, or the pCi-Neo empty plasmid and 2.5 μg pCi-Neo/Nef-Rluc and 2.5 μg pCi-Neo/CD4-EYFP; as a negative control, cells were transfected with 15 μg pCi-Neo, 2.5 μg pCi-Neo/Nef-Rluc, and 2.5 μg pHIS/β2AdrR-EYFP. Two days after transfection, BRET experiments were realized. After subtraction of the negative control spectrum from the sample one, the previously observed EYFP signal in cells expressing Nef-Rluc and CD4-EYFP was abrogated in the presence of the nonfluorescent receptors CD4 and CD4 414AA. (B) The same experiment was performed with the pCi-Neo/CD4 414AA-EYFP plasmid. The EYFP signal generated with Nef-Rluc/CD4 414AA-EYFP pair was not detected anymore when CD4 or CD4 414AA was coexpressed.

variants. These results indicate that the signals are specific to Nef and CD4 or CD4 414AA interactions.

In this work, for the first time, the direct interaction between the HIV-1 Nef protein and the human CD4 receptor was studied with wild-type proteins in human cells. By using the BRET technology, we directly detected the Nef-CD4 interaction in metabolically active cells and at physiological temperature. Surprisingly, we also observed the association of Nef with the CD4 414AA mutant. Although the fusion of Rluc and EYFP to Nef protein reduces its ability to down-regulate the membrane expression of CD4, and the CD4-EYFP fusion renders the receptor resistant to down-regulation, reproducible luminescence energy transfers between Nef-Rluc and CD4-EYFP or CD4 414AA-EYFP were detected. Compared to the BRET signal observed in prokaryotic cells as a result of homodimerization of circadian clock proteins (55), the signals revealing homo-oligomerization (4, 14, 36; our results obtained for the Nef dimerization) and hetero-oligomerization (36; our results obtained for the Nef-CD4 interaction) of proteins in eukaryotic cells are smaller, but remain sufficient to detect protein-protein interactions in human cells. The specificity of this weak signal could be questioned as an increased proximity

of CD4 and Nef could be due to lipid rafts localization of the two molecules. However, recent results strongly suggest that rafts do not significantly contribute to the effects of Nef on CD4 (48), although opposite conclusions were also reported (3). Moreover, one of the negative controls presented, β2Adr-EYFP, is also present in rafts, and energy transfer was not detected. A second transmembrane protein control also localized in rafts, CD8-EYFP, was used, and in the presence of Nef-Rluc, no BRET signal was measured (data not shown); however, the membrane concentration of CD8-EYFP being much lower than that of CD4-EYFP, it is thus difficult to compare these two conditions. As a complementary evaluation of the specificity of the Nef-CD4 interaction, competition experiments were done either with the wild-type CD4 or the CD4 414AA mutant and BRET signals were completely abolished. The fact that Nef-Rluc has a lower capacity to down-regulate CD4 and that CD4-EYFP cannot be down-regulated by Nef could have facilitated the detection of Nef/CD4 complex by BRET, maybe by increasing the Nef/CD4 complex half-life. Unfortunately, BRET could not be directly visualized by confocal microscopy, but we observed that, in our model Nef and the CD4 receptors colocalized at the plasma membrane, and the interactions are likely to occur at this site, as proposed previously (1, 11, 22, 38). Nevertheless, the existence of these interactions was confirmed by a different approach, using the coimmunoprecipitation method, not only with fusion proteins but also with full-length, wild-type proteins, indicating that complex formation is not restricted to the modified proteins. Our results clearly demonstrate that Nef and CD4 proteins interact in human cells.

Our data also confirm that the dileucine motif of the cytoplasmic domain of CD4 is critical for the Nef-induced CD4 downregulation. Nevertheless, we demonstrated that these residues are not implicated in the Nef/CD4 complex formation. This observation differs from those obtained in previous NMR experiments. However, data published during the final stage of our work (9) are in agreement with our result. Bentham et al. performed binding assays with a NefGST protein and full-length CD4 and CD4 414 receptors in Sf9 insect cells or in vitro. Although the NefGST did not induce CD4 down-modulation in Sf9 cells, the authors reported that NefGST formed a complex with CD4 and CD4 414AA. In the human HeLa cells, the NefGST protein was able to bind CD4 414AA but failed to form a stable complex with the wild-type CD4, most likely due to the transient nature of this interaction in these conditions. The results of this group and ours indicate that the dileucine motif of the CD4 cytoplasmic tail is not involved in the Nef-CD4 interaction. This motif is indispensable for the CD4 internalization and could be presumably implicated in the connection of the Nef/CD4 complex to the endocytic machinery. Our results show that the DSP cross-linker and the BRET technique allow us to observe this Nef-CD4 interaction in human cells. Thus, the experimental procedures we developed appear as efficacious systems to study the molecular bases of the Nef/CD4 complex formation.

The Nef-CD4 interaction might be an important step in Nef-mediated CD4 downregulation, which is a key function for this early viral protein. Nef has been shown to enhance HIV replication (21, 33) and to evolve during progression to AIDS, acquiring an augmented capacity to deregulate CD4 (12). Whether a concomitant modulation of Nef-CD4 interaction also takes place remains to be evaluated.

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REFERENCES

1. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane proximal CD4 cytoplasmic domain. Cell **76:**853–864.
2. **Alexander, L., Z. Du, M. Rosenzweig, J. U. Jung, and R. C. Desrosiers.** 1997. A role for natural simian immunodeficiency virus and human immunodeficiency virus type 1 *nef* alleles in lymphocyte activation. J. Virol. **71:**6094–6099.
3. **Alexander, M., Y. Bor, K. S. Ravichandran, M.-L. Hammarsjöld, and D. Rekosh.** 2004. Human immunodeficiency virus type 1 Nef associates with lipid rafts to downmodulate cell surface CD4 and class I major histocompatibility complex expression and to increase viral infectivity. J. Virol. **78:** 1685–1696.
4. **Angers, S., A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis, and M. Bouvier.** 2000. Detection of β2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc. Natl. Acad. Sci. USA **97:**3684–3689.
5. **Arold, S., F. Hoh, S. Domergue, C. Birk, M. A. Delsuc, M. Jullien, and C. Dumas.** 2000. Characterization and molecular basis of the oligomeric structure of HIV-1 Nef protein. Protein Sci. **9:**1137–1148.
6. **Bell, I., T. M. Schaefer, R. P. Trible, A. Amedee, and T. A. Reinhart.** 2001. Down-modulation of the costimulatory molecule, CD28, is a conserved activity of multiple SIV Nefs and is dependent on histidine 196 of Nef. Virology **283:**148–158.
7. **Bell, I., C. Ashman, J. Maughan, E. Hooker, F. Cook, and T. A. Reinhart.** 1998. Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) zeta chain leads to TCR down-modulation. J. Gen. Virol. **79:**2717–2727.
8. **Benichou, S., M. Bomsel, M. Bodeus, H. Durand, M. Doute, F. Letourneur, J. Camonis, and R. Benarous.** 1994. Physical interaction of the HIV-1 Nef protein with beta-COP, a component of non-clathrin-coated vesicles essential for membrane traffic. J. Biol. Chem. **269:**30073–30076.
9. **Bentham, M., S. Mazaleyrat, and M. Harris.** 2003. The di-leucine motif in the cytoplasmic tail of CD4 is not required for binding to human immunodeficiency virus type 1 Nef, but is critical for CD4 down-modulation. J. Gen. Virol. **84:**2705–2713.
10. **Boute, N., K. Pernet, and T. Issad.** 2001. Monitoring the activation state of the insulin receptor using bioluminescence resonance energy transfer. Mol. Pharmacol. **60:**640–645.
11. **Bresnahan, P. A., W. Yonemoto, S. Ferrell, D. Williams-Herman, R. Geleziunas, and W. C. Greene.** 1998. A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. Curr. Biol. **8:**1235–1238.
12. **Carl, S., T. C. Greenough, M. Krumbiegel, M. Greenberg, J. Skowronski, J. L. Sullivan, and F. Kirchhoff.** 2001. Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS. J. Virol. **75:**3657–3665.
13. **Chen, Y.-L., D. Trono, and D. Camaur.** 1998. The proteolytic cleavage of human immunodeficiency virus type 1 Nef does not correlate with its ability to stimulate virion infectivity. J. Virol. **72:**3178–3184.
14. **Cheng, Z.-J., and L. J. Miller.** 2001. Agonist-dependent dissociation of oligomeric complexes of G protein-coupled cholecystokinin receptors demonstrated in living cells using bioluminescence resonance energy transfer. J. Biol. Chem. **276:**48040–48047.
15. **Chowers, M. Y., C. A. Spina, T. J. Kwoh, N. J. S. Fitch, D. D. Richman, and J. C. Guatelli.** 1994. Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact *nef* gene. J. Virol. **68:**2906–2914.
16. **Craig, H. M., T. R. Reddy, N. L. Riggs, P. P. Dao, and J. C. Guatelli.** 2000. Interactions of HIV-1 nef with the mu subunits of adaptor protein complexes 1, 2, and 3: role of the dileucine-based sorting motif. Virology **271:**9–17.
17. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, and C. Chatfield.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
18. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature **350:**508–511.
19. **Geleziunas, R., W. Xhu, K. Takeda, H. Ichijo, and W. C. Green.** 2001. HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infecting host cell. Nature **410:**834–838.

20. **Geyer, M., H. Yu, R. Mandic, T. Linnemann, Y. H. Zheng, O. T. Fackler, and B. M. Peterlin.** 2002. Subunit H of the V-ATPase binds to the medium chain of AP-2 and connects Nef to the endocytic machinery. J. Biol. Chem. **277:** 28521–28529.
21. **Glushakova, S., J. Münch, S. Carl, T. C. Greenough, J. L. Sullivan, L. Margolis, and F. Kirchhoff.** 2001. CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with $CD4^+$ T-cell depletion in human lymphoid tissue ex vivo. J. Virol. **75:**10113–10117.
22. **Greenberg, M. E., S. Bronson, M. Lock, M. Neumann, G. N. Pavlakis, and J. Skowronski.** 1997. Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. **16:**10113–10117.
23. **Grzesiek, S., S. J. Stahl, P. T. Wingfield, and A. Bax.** 1996. The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. Biochemistry **35:**10256–10261.
24. **Harris, M. P., and J. C. Neil.** 1994. Myristoylation-dependent binding of HIV-1 Nef to CD4. J. Mol. Biol. **241:**136–142.
25. **Hua, J., W. Blair, R. Truant, and B. R. Cullen.** 1997. Identification of regions in HIV-1 Nef required for efficient downregulation of cell surface CD4. Virology **231:**231–238.
26. **Janvier, K., H. Craig, S. Le Gall, R. Benarous, J. Guatelli, O. Schwartz, and S. Benichou.** 2001. Nef-induced CD4 downregulation: a diacidic sequence in human immunodeficiency virus type 1 Nef does not function as a protein sorting motif through direct binding to β-COP. J. Virol. **75:**3971–3976.
27. **Jason, J., and K. L. Inge.** 2001. Modulation of CD8 and CD3 by HIV or HIV antigens. J. Immunol. **53:**259–267.
28. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–652.
29. **Kienzle, N., J. Freund, H. R. Kalbitzer, and N. Mueller-Lantzsch.** 1993. Oligomerization of the Nef protein from human immunodeficiency virus (HIV) type 1. Eur. J. Biochem. **214:**451–457.
30. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
31. **Liu, X. L., N. Heveker, O. T. Fackler, S. Arold, S. Le Gall, K. Janvier, B. M. Peterlin, C. Dumas, O. Schwartz, S. Benichou, and R. Benarous.** 2000. Mutation of a conserved residue (D123) required for oligomerization of human immunodeficiency virus type 1 Nef protein abolishes interaction with human thioesterase and results in impairment of Nef biological functions. J. Virol. **74:**5310–5319.
32. **Lock, M., M. E. Greenberg, A. J. Iafrate, T. Swigut, J. Muench, F. Kirchhoff, N. Shohdy, and J. Skowronski.** 1999. Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis. EMBO J. **18:**2722–2733.
33. **Lundquist, C. A., M. Tobiume, J. Zhou, D. Unutmaz, and C. Aiken.** 2002. Nef-mediated downregulation of CD4 enhances human immunodeficency virus type 1 replication in primary T lymphocytes. J. Virol. **76:**4625–4633.
34. **Mandic, R., O. T. Fackler, M. Geyer, T. Linnemann, Y. H. Zheng, and B. M. Peterlin.** 2001. Negative factor from SIV binds to the catalytic subunit of the V-ATPase to internalize CD4 and to increase viral infectivity. Mol. Biol. Cell **12:**463–473.
35. **Mangasarian, A., V. Piguet, J.-K. Wang, Y.-L. Chen, and D. Trono.** 1999. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. J. Virol. **73:**1964–1973.
36. **McVey, M., D. Ramsay, E. Kellett, S. Rees, S. Wilson, A. J. Pope, and G. Milligan.** 2001. Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. J. Biol. Chem. **276:**14092–14099.
37. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp Med. **179:**101–113.
38. **Piguet, V., Y.-L. Chen, A. Mangasarian, M. Foti, J.-L. Carpentier, and D. Trono.** 1998. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the μ chain of adaptor complexes. EMBO J. **17:**2472–2481.
39. **Piguet, V., F. Gu, M. Foti, N. Demaurex, J. Gruenberg, J.-L. Carpentier, and D. Trono.** 1999. Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targetting signal through the binding of β-COP in endosomes. Cell **97:**63–73.
40. **Poulin, L., and J. A. Levy.** 1992. The HIV-1 nef gene product is associated with phosphorylation of a 46 kD cellular protein. AIDS **6:**787–791.
41. **Preusser, A., L. Briese, A. Baur, and D. Willbord.** 2001. Direct in vitro binding of full-length human immunodeficiency virus type 1 Nef protein to CD4 cytoplasmic domain. J. Virol. **75:**3960–3964.
42. **Rossi, F., A. Gallina, and G. Milanesi.** 1996. Nef-CD4 physical interaction sensed with the yeast two-hybrid system. Virology **217:**397–403.
43. **Salvi, R., A. R. Garbuglia, A. Di Caro, S. Pulciani, F. Montella, and A. Benedetto.** 1998. Grossly defective *nef* gene sequences in a human immunodeficiency virus type 1 seropositive long-term nonprogressor. J. Virol. **72:** 3646–3657.
44. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1539–1543.
45. **Schaeffer, E., R. Geleziunas, and W. C. Greene.** 2001. Human immunodeficiency type 1 Nef functions at the level of virus entry by enhancing cytoplasmic delivery of virions. J. Virol. **75:**2993–3000.
46. **Schwartz, O., V. Marechal, S. Le Gall, F. Lemmonier, and J.-M. Heard.** 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. **2:**338–341.
47. **Simmons, A., V. Aluvihare, and A. McMichael.** 2001. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity **14:**763–777.
48. **Sol-Foulon, N., C. Esnault, Y. Pecherancier, F. Porrot, P. Metais-Cunha, F. Bachelerie, and O. Schwartz.** 2004. The effects of Nef on CD4 surface expression and viral infectivity in lymphoid cells are independent of rafts. J. Biol. Chem. **279:**31398–31408.
49. **Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman.** 1994. The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. **179:**115–123.
50. **Swigut, T., N. Shohdy, and J. Skowronski.** 2001. Mechanism for down-regulation of CD28 by Nef. EMBO J. **20:**1593–1604.
51. **Valenzuela-Fernandez, A., T. Palanche, A. Amara, A. Magerus, R. Altmeyer, T. Delaunay, J. L. Virelizier, F. Baleux, J. L. Galzi, and F. Arenzana-Seisdedos.** 2001. Optimal inhibition of X4 HIV isolates by the CXC chemokine stromal cell-derived factor 1 alpha requires interaction with cell surface heparan sulfate proteoglycans. J. Biol. Chem. **276:**26550–26558.
52. **Vollmer, J. Y., P. Alix, A. Chollet, K. Takeda, and J. L. Galzi.** 1999. Subcellular compartimentalization of activation and desensitization of responses mediated by NK2 neurokinin receptors. J. Biol. Chem. **274:**37915–37922.
53. **Willard-Gallo, K. E., M. Furtado, A. Burny, and S. M. Wolinsky.** 2001 Down-modulation of TCR/CD3 surface complexes after HIV-1 infection is associated with differential expression of the viral regulatory genes. Eur. J. Immunol. **31:**969–979.
54. **Wolf, D., V. Witte, B. Laffert, K. Blume, E. Stromer, S. Trapp, P. D'Aloja, A. Schürmann, and A. S. Baur.** 2001. HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. Nat. Med. **7:**1217–1224.
55. **Xu, Y., D. W. Piston, and C. H. Johnson.** 1999. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc. Natl. Acad. Sci. USA **96:**151–156.
56. **Zhou, J., and C. Aiken.** 2001. Nef enhances human immunodeficiency virus type 1 infectivity resulting from intervirion fusion: evidence supporting a role for Nef at the virion envelope. J. Virol. **75:**5851–5859.