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Human Immunodeficiency Virus Type 1 Nef Activates p21-Activated Kinase via Recruitment into Lipid Rafts

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The Nef protein of human immunodeficiency virus type 1 is an important factor in AIDS pathogenesis. In addition to downregulating CD4 and major histocompatibility complex class I molecules from the cell surface, as well as increasing virion infectivity, Nef triggers activation of the T-cell receptor (TCR) cascade to facilitate virus spread. Signaling pathways that are induced by Nef have been identified; however, it is unclear how and in which subcellular compartment Nef triggers signaling. Nef recruits a multiprotein complex to activate the cellular Pak kinase that mediates downstream effector functions. Since a subpopulation of Nef is present in detergent-insoluble microdomains (lipid rafts) from where physiological TCR signaling is initiated, we tested whether lipid rafts are instrumental for Nef-mediated Pak activation. In flotation analysis, Nef-associated Pak activity exclusively fractionated with lipid rafts. Activation of Pak in the presence of Nef coincided with lipid raft recruitment of the kinase, which was otherwise excluded from detergent-insoluble microdomains. Experimental solubilization of lipid rafts interfered with the association of Pak activity with Nef. To analyze the importance of the raft localization for Nef function more rigorously, we generated a palmitoylated Nef (PalmNef). PalmNef was highly enriched in lipid rafts and associated with significantly higher levels of Pak activity than Nef. Notably, activation of Pak by its physiological activators, Cdc42 and Rac, also occurred in lipid rafts and required raft integrity. Together, these data suggest that Nef induces signal transduction via the recruitment of a signaling machinery including Pak into lipid rafts, thereby mimicking a physiological cellular mechanism to initiate the TCR cascade.

The accessory gene product Nef is a major determinant for the pathogenesis of the primate lentiviruses human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV). Viral isolates lacking functional *nef* genes grow to low titers in vivo and induce no or delayed disease progression (16, 33). In addition, expression of Nef is sufficient to cause an AIDS-like disease in a transgenic mouse model (29). This impact on the outcome of HIV/SIV infection likely results from the synergy of multiple functions exerted by Nef that may be differentially regulated over time (12). N-terminal myristoylation that mediates the localization of Nef to intracellular and plasma membranes is essential for all known effects of the viral protein. Since Nef lacks any enzymatic activity, its functions are mediated by the interaction with a number of cellular ligands (26). First, Nef targets a set of cell surface receptors and connects them to the endocytic machinery, thereby affecting their intracellular sorting routes (27, 28, 36, 42, 51). Among these receptors, CD4 and major histocompatibility complex class I (MHC-I) molecules are best studied. Although CD4 is targeted to lysosomal degradation by Nef (1), MHC-I molecules are routed to the *trans*-Golgi network in a stepwise mechanism (8, 52, 64). Whereas downregulation of the primary viral receptor CD4 is thought to prevent superinfection and to counteract negative effects of CD4 on particle release and infectivity (7, 24, 34, 57), internalization of MHC-I molecules leads to the escape of cytotoxic-T-lymphocyte (CTL) recognition of infected cells (14). Second, Nef enhances virion infectivity in a single round of replication, presumably by modulating the lipid composition of the viral envelope (2, 63, 76). Finally, Nef alters a variety of cellular signal transduction pathways. Since HIV does not replicate in resting cells (67, 74), activation of target cells should be of great benefit for viral spread in the host. Importantly, Nef-mediated increase of the cellular activation state can be detected in target T cells already prior to integration of the incoming viral genome (72). Such activation was recently shown to be relevant in a primary cell coculture model of immature dendritic cells and autologous T cells, where efficient virus replication requires Nef and correlates with its ability to induce signaling (25, 45, 50).

Depending on the nature of the target cell, various signaling cascades are targeted by Nef. In macrophages, Nef activates the Hck kinase resulting in the release of T-cell attracting soluble factors (68). When expressed in T cells directly, Nef induces an intermediate state of activation via the TCR that requires an additional stimulus to reach permissivity for HIV replication (21, 62, 65). At least two multiprotein complexes are involved in this activation of the TCR cascade. NAKC, a protein complex containing several kinases, causes activation of Lck, possibly providing a TCR proximal activation signal (6). In addition, Nef associates with the cellular serine/threonine p21-activated kinase (Pak) (48, 59, 61). Among the Pak family, Pak1 and Pak2 (henceforth Pak) can associate with Nef, with Pak2 being the preferentially recruited Pak isoform (3, 22, 53). This interaction can be readily detected when Nef-associated kinase activity is being scored, i.e., when the autophosphorylation of Pak is revealed. Detection of the Nef-Pak

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interaction by less-sensitive methods such as Western blotting, however, has remained difficult, suggesting that the interaction is transient and that Nef associates with a highly active subpopulation of Pak (3, 54). This is also consistent with the idea that Nef increases Pak activity, which was demonstrated by some but not all investigators (3, 48, 54, 61). The mechanism and subcellular localization of Nef-mediated activation of Pak is unknown except that it requires the association of Nef with cellular membranes (60) and involves assembly of a 1 MDa multiprotein complex (22). With the SH3 binding domain (72PxxP75) and two arginine residues or one lysine and one arginine (105R/KR106), respectively, two highly conserved interaction surfaces are required for the association of Nef with Pak activity. Our previous studies suggested that Nef recruits and activates the guanine exchange factor Vav via the PxxP motif and that the R/KR motif is involved in the direct recruitment of the Pak kinase (22, 23). Via the activation of Vav, Nef would induce the activity of the GTPases Rac and/or Cdc42, whose activity is required for the activation of Pak by Nef (41).

Activation of Pak by Nef has multiple functional consequences that optimize virus production, including antiapoptotic effects (71), as well as induction of the viral promoter and cytoskeletal rearrangements (22, 23). Importantly, the PxxP motif of Nef that is a prerequisite for Pak activation is required for the optimal spread of HIV in primary cells (25, 44, 58). Furthermore, one study reported a correlation between the association of Nef with Pak activity and disease progression in SIV-infected macaques (61). A similar study yielded contrasting results; however, the fast disease progression of the animals used might have obscured the requirements for cell activation by Nef (35). This has been revisited more recently in Nef-transgenic mice, where the association of Nef with active Pak is readily observed and correlates with development of disease (29, 30). Together, these results implicate the activation of Pak by Nef as one important determinant for its role as a pathogenicity factor in vivo.

Activation of Pak kinases in general is a complex, multistep process. Increasing evidence suggests that Pak is present as a homodimer of two autoinhibited Pak molecules in the inactive state (11, 37, 49). Interaction with specific activators such as Rac/Cdc42 GTPases or sphingosine releases autoinhibition and allows for subsequent stepwise phosphorylation events that mediate the transition to a fully active conformation (4, 15). These involve phosphorylation events on serine/threonine as well as tyrosine residues (13, 55, 75). On the cellular level, membrane localization strongly increases Pak activity via a GTPase-dependent mechanism (17, 39, 40). Translocation of Pak to the plasma membrane appears to depend on its interaction with tethering factors such as the adaptor molecule Nck (39). The TCR cascade represents one example of surface receptor mediated signal transduction inducing the activity of Pak, where kinase activation involves the recruitment of a Nck-Pak module to the plasma membrane (10). How membrane targeting of Pak results in its activation, however, is unclear.

Lipid rafts are lateral heterogeneities within cellular membranes enriched in cholesterol and sphingolipids, as well as a specific set of raft proteins that are defined by their insolubility in cold detergents such as Triton X-100. These microdomains have been recognized as central platforms for cellular transport and signal transduction processes (32, 66). This concept applies for the TCR cascade, wherein signaling is initiated by the recruitment of second messenger molecules into lipid rafts of the plasma membrane. It is believed that this concentration of signaling molecules facilitates the generation of a TCR signal, an effect that is enforced and sustained by cytoskeleton mediated clustering of rafts upon TCR engagement (19, 20). Although the raft recruitment of factors acting upstream of Pak such as Vav and Lck has been reported to be critical for TCR signaling (31, 47, 73), no information is available on the lipid raft incorporation of Pak itself. Small populations of Nef are found in lipid rafts in stably Nef-expressing human T-cell lines and in fibroblasts expressing HIV proviral DNA (69, 70, 76). These results, together with the low abundance of the Nef-Pak complex in cells, raised the possibility that Nef, in mimicry of cellular mechanisms, recruits signaling intermediates into lipid rafts to initiate signal transduction. This hypothesis was tested in the present study.

Here we show that Nef activates Pak via the recruitment into lipid rafts. Nef-associated Pak activity was detected exclusively in lipid raft fractions and Nef recruited Pak into plasma membrane rafts. Consequently, disruption of rafts interfered with the activation of the kinase by Nef. Furthermore, enhanced raft targeting of a palmitoylated Nef resulted in an increase in Nef-associated Pak activity. Importantly, activation of Pak by Cdc42 and Rac also occurred in rafts and was dependent on raft integrity. Together, these data suggest that raft recruitment is an essential step in the activation of Pak by Nef. Furthermore, these results identify raft recruitment as a general mechanism of Pak activation.

MATERIALS AND METHODS

Cells and reagents. 293T and Jurkat Tag cells (Jurkat cells with the large T antigen of simian virus 40) (23) were maintained in Dulbecco modified Eagle medium and RPMI 1640 medium, respectively, supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. The following antibodies were used: monoclonal antibody (MAb) anti-HA (clone F-7), MAb anti-CD45 (clone 35-Z6), polyclonal rabbit anti-hemagglutinin antibody (HA), and anti-glutathione *S*-transferase (GST) antibody (all from Santa Cruz Biotechnology, Inc., Heidelberg, Germany), MAb anti-CD59 clone H19 (BD Pharmingen, Heidelberg, Germany), MAb anti-transferrin receptor (TfR) clone H68.4 (Zymed, Berlin, Germany), polyclonal rabbit anti-CTx antibody (Sigma, Munich, Germany), and polyclonal rabbit anti-AU-1 antibody (BAbCO, Richmond, Calif.). Polyclonal rabbit serum against green fluorescent protein (GFP) was kindly provided by Hans-Georg Kräusslich. The polyclonal rabbit serum against Nef was derived against a peptide encompassing amino acids 60 to 73 of HIV-1_{NL4-3} Nef. Horseradish peroxidase (HRP)-conjugated cholera toxin (CTx) B subunit, a protease inhibitor cocktail, and octyl-glucopyranoside (OTG) were purchased from Sigma. Alexa 594-conjugated CTx was from Molecular Probes (Leiden, The Netherlands), as well as all secondary fluorescent antibodies.

Expression plasmids. Nef from HIV-1–SF2 was used throughout the study. Plasmids encoding Nef.GFP and G2ANef.GFP, as well as AU1-tagged Nef, were described earlier (22, 25). The expression plasmid for PalmNef.GFP was generated by PCR with mutagenic oligonucleotides introducing a G3C mutation into Nef.GFP. Plasmids encoding PAK2D125A-HA (also carrying an additional His tag) and Cdc42V12.GST were kindly provided by Kalle Saksela (55). The D125A mutation in Pak2 results in an intermediate state of activation that increases its susceptibility for activation by Rac/Cdc42 and Nef. The RacL61.GFP and Cdc42L61.GFP expression constructs were kindly provided by Michael Way.

Raft fractionation. 293T and Jurkat Tag cells were transfected with Lipofectamine (Gibco/Life Technologies, Eggenstein, Germany) or electroporation, respectively. At 24 h posttransfection, cells were lysed in ice-cold TXNE (1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, protease inhibitors) and incubated for 20 min on ice. After homogenization by 15 strokes with a Dounce homogenizer, lysates were adjusted to 40% Optiprep (Life Tech-

nologies, Karlsruhe, Germany) and deposited in SW60 Ti centrifuge tubes. Samples were overlaid with 2.5 ml of 28% Optiprep in TXNE and with 0.6 ml of TXNE and then centrifuged for 3 h at 35,000 rpm at 4°C. Eight fractions of 500 μl were collected from the top. Aliquots (15 μl) of each fraction were analyzed by Western blotting. In some experiments, only raft and nonraft fractions were analyzed. Based on sedimentation of raft and nonraft markers, fraction 2 was identified as the raft fraction, whereas the nonraft fraction was derived by pooling fractions 7 and 8, and equal volumes of both fractions were analyzed. To disrupt lipid rafts, 50% of each lysate was incubated with 60 mM OTG for 20 min at 4°C prior to flotation analysis and compared to the remaining of the lysate without OTG treatment.

IVKA. In vitro kinase assay (IVKA) from total lysates was performed as described earlier (22). Briefly, 5×10^6 cells expressing Nef.GFP were lysed in KEB (137 mM NaCl, 50 mM Tris-HCl [pH 8], 2 mM EDTA, 0.5% Nonidet P-40, Na_3VO_4, protease inhibitors) at 48 h posttransfection. Cleared lysates were immunoprecipitated with anti-GFP antibodies. After an intensive wash in KEB, the immunoprecipitates were resuspended in KAB (50 mM HEPES [pH 8], 150 mM NaCl, 5 mM EDTA, 0.02% Triton X-100, 10 mM $MgCl_2$) containing 10 μCi of [γ-^{32}P]ATP (Amersham, Freiburg, Germany) per reaction. After incubation for 5 min, samples were washed and bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography. Quantification was performed on a phosphorimager (Bio-Rad, Munich, Germany) by using QuantityOne software (Bio-Rad). For IVKA from lipid raft fractionations, 250-μl aliquots of pooled raft and nonraft fractions were diluted fivefold in TXNE and subjected to a kinase reaction as described for total lysates.

Western blotting. For Western blot analysis, samples were boiled in SDS sample buffer, separated by SDS–10% PAGE, and transferred to a nitrocellulose membrane. Protein detection was performed after incubation with appropriate first and secondary antibodies by using the Super-Signal Pico detection kit (Pierce, Bonn, Germany) according to the manufacturer's instructions. Detection of GM1 was performed on polyvinylidene difluoride membranes with 100 ng of HRP-conjugated CTx/ml.

Raft patching and confocal microscopy. At 48 h posttransfection, Jurkat Tag cells were incubated with 25 μg of Alexa 594-conjugated CTx/ml in 0.1% bovine serum albumin–phosphate-buffered saline (PBS) for 30 min on ice. Cross-linking was performed by incubation with anti-CTx antibody at a 1:200 dilution for 30 min at 4°C and for 10 min at 37°C. Cells were seeded on poly-L-lysine-coated coverslips and fixed with 3% paraformaldehyde-PBS. For two-color analysis, images were obtained with a confocal laser scanning microscope (Leica TCS-NT System) attached to a DM IRB inverted microscope with a PLAPO 63×1.32 oil immersion objective lens. For three-color analysis, cells were permeabilized and stained with the anti-HA antibody, and the appropriate Alexa 350-conjugated secondary antibody. Images were then obtained with a Leica SP2 scanner and a DMRE microscope. All images were processed by using Adobe Photoshop.

Metabolic labeling. Eighty percent confluent six-well plates of 293T cells expressing Nef.GFP fusion proteins were serum starved for 1 h at 24 h posttransfection. After a wash with PBS, the cells were metabolically labeled with 50 μCi of ^{35}S In Vitro Cell Labeling Pro Mix (Amersham) or 150 μCi of [9,10(*n*)-^{3}H]palmitic acid (Amersham) for 4 h. Palmitic acid solubilized in ethanol was air dried and resuspended in 10 μl of dimethyl sulfoxide prior to cell labeling. After the labeling period, cells were washed in PBS and lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.2], 1% Triton X-100, 0.1% SDS, protease inhibitors), and cleared lysates were subjected to anti-GFP immunoprecipitation. After SDS-PAGE of the immunoprecipitates, gels were fixed and enhanced by using Amplify (Amersham) and exposed for 24 h (^{35}S) and 6 weeks (^{3}H), respectively, for autoradiography.

RESULTS

Nef-associated Pak activity fractionates exclusively in lipid rafts. To address the question of whether lipid rafts play a role in the activation of Pak by Nef, we first investigated whether Nef-associated Pak activity can be detected in lipid rafts. A fusion protein between Nef from the HIV-1 strain SF2 and GFP was used to allow direct comparison of biochemical and immunohistological data. Raft flotation analysis was performed from 293T cells transiently expressing Nef.GFP and HA-tagged Pak2 resulting in eight fractions (Fig. 1A). Western blot analysis revealed that the raft marker GM1 exclusively fractionated in fraction 2, whereas the nonraft marker TfR was found in fractions 7 and 8. Due to the low sensitivity of GM1 detection by Western blotting, the sphingolipid is only detected upon concentration in lipid rafts (see, for example, reference 46). Thus, fraction 2 was defined as the raft fraction, while fractions 7 and 8 represent the nonraft portion of the lysates. In agreement with previous reports in which Nef was expressed in stable cell lines or from the HIV-1 provirus (69, 70, 76), small amounts of Nef protein could be detected in lipid rafts, with the large majority of the protein residing in nonraft fractions (Fig. 1A, WB). Pak2 protein displayed a similar distribution, with only a minor fraction of the protein residing in lipid rafts. Of the two isoforms of Pak2 detected in 293T cells in some but not all experiments possibly representing different phosphorylation states of the kinase, only the faster-migrating Pak2 isoform was present in lipid raft fractions. When the individual fractions were assayed for Nef-associated kinase activity after immunoprecipitation with the anti-GFP antibody, a phosphorylated band of ~65 kDa was observed that reflects autophosphorylation of epitope-tagged, Nef-associated Pak2 (3, 52, 53). This phosphorylation event was found in the total cell lysate and in lipid rafts but was absent from nonraft fractions (Fig. 1A, IVKA). Thus, only the minor Nef and Pak2 populations in lipid rafts could functionally interact, whereas the robust levels of both proteins in nonraft fractions failed to produce detectable amounts of Nef-associated Pak activity. To exclude the possibility that these results were affected by the GFP moiety of the fusion proteins used, the distribution of Nef-associated Pak activity was compared between Nef.GFP and a Nef protein that only carries a six-amino-acid AU-1 tag at its C terminus. For simplicity, only the fractions relevant to the present study (fraction 2 = R [rafts]; pooled fractions 7 and 8 = NR [nonrafts]) were subjected to anti-GFP or anti-AU-1 immunoprecipitation, respectively, followed by an in vitro kinase reaction (Fig. 1B). In both cases, Nef-associated Pak activity was readily detected in the raft fraction but was absent in nonrafts. These results suggested that, similar to other activities of Nef (22, 28, 38), Nef.GFP fusion proteins are functional homologues of Nef when the association with Pak activity is measured. We next repeated this experiment in relevant HIV target Jurkat T cells scoring for the activity of endogenous Pak associated with Nef.GFP (Fig. 1C). Similar to 293T cells, the lipid raft marker GM1 floated in the raft fraction (fraction 2, R), and the nonraft marker TfR was present in the nonraft fractions (pooled fractions 7 and 8, NR). Upon IVKA reaction after anti-GFP immunoprecipitation, two distinct signals reflecting autophosphorylated Pak and another unidentified T-cell specific phosphoprotein of higher apparent molecular weight were readily detected in the raft fraction and the total lysate (L). Although displaying elevated nonspecific background, these specific phosphoproducts were absent in the nonraft fraction. These results therefore confirmed our previous observation in 293T cells that Nef-associated Pak2 activity is exclusively found in lipid raft fractions. As a control for specificity, no kinase activity was detected after anti-GFP immunoprecipitation from GFP-expressing Jurkat (Fig. 1D) or 293T (data not shown) cells. Of note, the Nef-associated Pak2 activity was consistently stronger in the raft fraction than in the total cell lysate in 293T cells, whereas no significant difference was observed in Jurkat cells (compare Fig. 1A and C). This

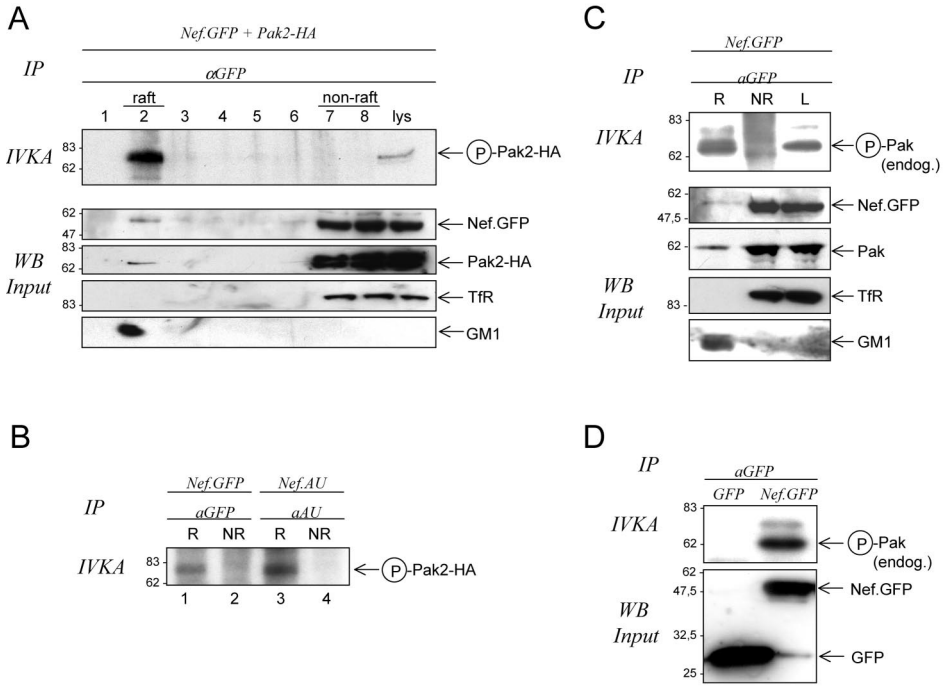

FIG. 1. Exclusive fractionation of Nef.GFP-associated Pak activity in lipid rafts. (A) Lipid raft flotation analysis from transfected 293T cells expressing Nef.GFP and Pak2-HA. Cell lysates (1% Triton X-100) were separated by Optiprep gradient ultracentrifugation, and eight fractions were collected from the top (fraction 1) to the bottom (fraction 8) of the gradient and analyzed, together with the unfractionated cell lysate (lys). Equal volumes of each fraction were subjected to either IVKA after anti-GFP immunoprecipitation or Western blotting to detect the indicated proteins or GM1, respectively. (B) IVKA from a flotation analysis of the raft fraction (R, fraction 2) and the pooled nonraft fractions (NR, fractions 7 and 8) from 293T cells expressing Nef.GFP (lanes 1 and 2) or Nef.AU-1 (lanes 3 and 4). IVKA was performed after anti-GFP or anti-AU1 immunoprecipitation, respectively. (C) IVKA and Western blotting from a flotation analysis of the raft fraction (R, fraction 2) and the pooled nonraft fractions (NR, fractions 7 and 8) from Jurkat cells expressing Nef.GFP. IVKA was performed after anti-GFP immunoprecipitation. (D) IVKA and anti-GFP Western blotting from total cell lysates of Jurkat cells expressing GFP or Nef.GFP, respectively. IVKA was performed after anti-GFP immunoprecipitation. WB, Western blotting.

finding might reflect differences between the 293T and Jurkat cells used, wherein the relatively low levels of Nef-associated Pak2 activity in the total lysates of 293T cells are enhanced upon raft aggregation, whereas the robust Pak activity in Nef-expressing Jurkat cells is not further increased. Together, we conclude that the Nef-associated Pak activity present in total cell lysates can be attributed to the small fraction of Nef and Pak proteins incorporated into lipid rafts.

Nef is present in raft clusters in T cells. We next visualized the incorporation of Nef into lipid rafts in intact cells. Jurkat T cells transiently expressing various Nef.GFP fusion proteins were incubated with Alexa 594-conjugated CTx that binds to the raft sphingolipid GM1, and lipid rafts were clustered with the anti-CTx antibody (31) (Fig. 2). Confocal microscopy revealed that this raft clustering resulted in the formation of large GM1 containing patches at the plasma membrane (CTx 594 panels). For Nef.GFP, two distinct subcellular localizations were observed following raft clustering. Significant amounts of Nef.GFP were diffusely distributed throughout the cytoplasm. Another population of Nef.GFP however was found at the plasma membrane, where a marked accumulation in distinct patches was observed (see row A, GFP panel). Importantly, a significant fraction of these membrane patches colocalized with lipid raft aggregates indicated by the CTx staining (see row A, merge panel). In addition, areas with a more diffuse staining of Nef.GFP were also detected at the plasma membrane, which did not colocalize with CTx. In marked contrast, the nonmyristoylated G2A mutant of Nef was exclusively localized diffusely within the cytoplasm and did not accumulate at CTx clusters at the plasma membrane (Fig. 2C). These results were in agreement with the biochemical fractionation data that revealed the myristoylation-dependent incorporation of a subpopulation of Nef into lipid rafts (see Fig. 1A and C and 4C). The localization of PalmNef.GFP (Fig. 2B) will be discussed below. As controls, efficient raft clustering of CD59 and the insensitivity of CD45 toward the clustering of lipid rafts (31) were monitored (Fig. 2D and E). The distribution of these markers was unaffected by the presence of Nef (data not shown). These results confirm the biochemical data demonstrating the incorporation of Nef in Triton X-100-insoluble microdomains and reveal that a subpopulation of Nef resides in lipid rafts at the plasma membrane.

Lipid raft integrity is a prerequisite for the activation of Pak by Nef. We next sought to investigate whether lipid raft incorporation of Nef and Pak is required for the activation of the kinase by Nef. We therefore analyzed the effects of raft disruption on the in vitro kinase reaction. 293T cells transiently expressing Pak2 and Nef.GFP were subjected to raft flotation analysis and pooled raft versus nonraft fractions were analyzed (Fig. 3, R versus NR, respectively). To disrupt lipid rafts, half of the cell lysates were treated with the detergent OTG at 60 mM for 20 min prior to flotation analysis. OTG is widely used

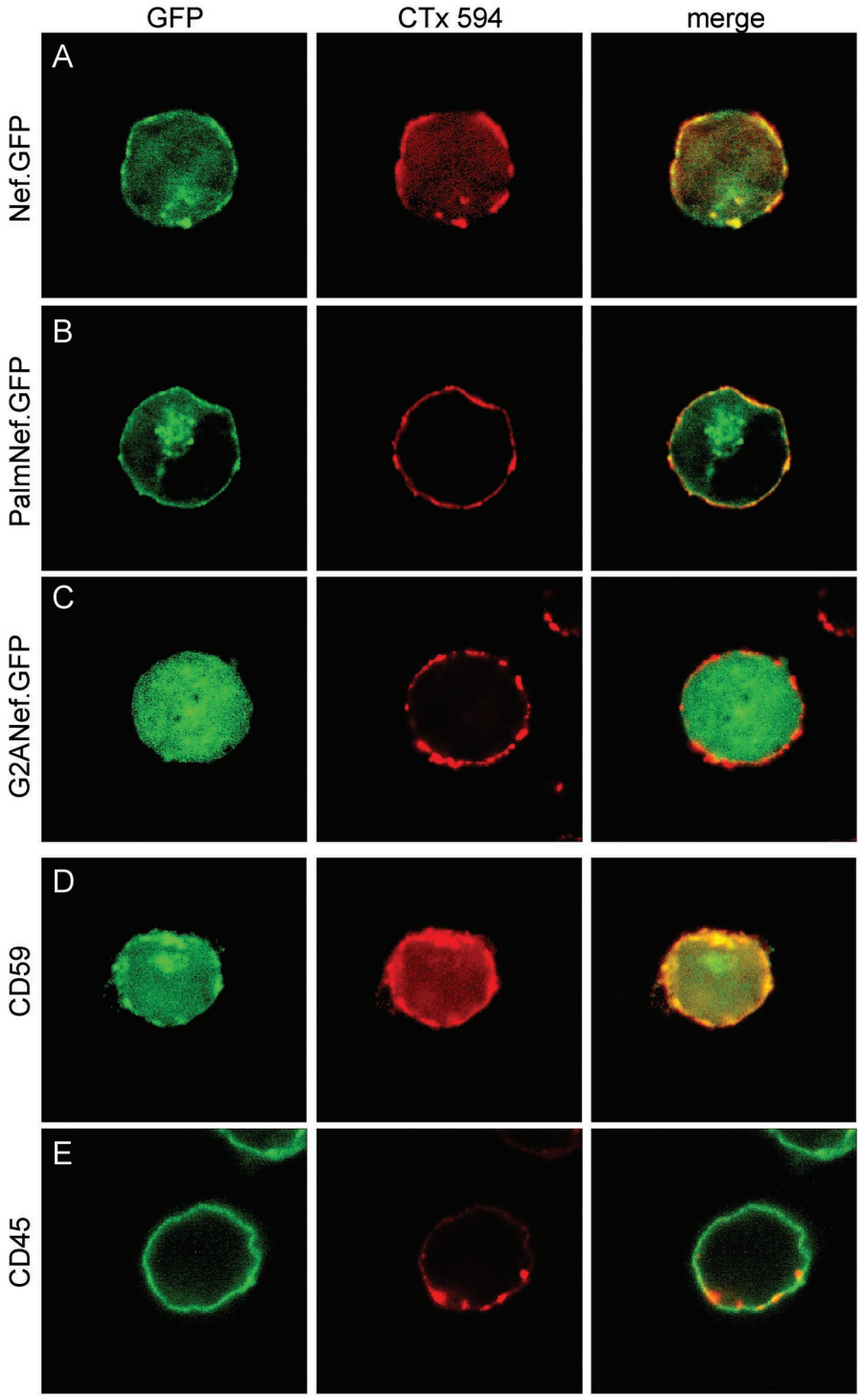

FIG. 2. Clustering of Nef.GFP with lipid rafts. (A to C) Jurkat cells expressing the indicated Nef.GFP fusion proteins were subjected to raft clustering by incubation with Alexa 594-conjugated CTx and subsequent cross-linking with the anti-CTx antibody. GFP and Alexa 594 fluorescence, respectively, was analyzed by confocal microscopy. The merge panel depicts the overlay of both fluorescence channels. Single representative sections are presented. (D and E) Controls of nontransfected cells after CTx-Alexa 594 raft clustering and staining with the fluorescein isothiocyanate-conjugated anti-CD59 and anti-CD45 antibodies, respectively.

to solubilize raft-associated protein complexes (5, 9, 46). To exclude that OTG solubilization triggers migration of the Nef-Pak2 complex to nonraft fractions other than 7 and 8, fractions 3 to 8 were pooled in this experiment and are referred to as NR fractions. Western blot analysis revealed that OTG treatment was effective as indicated by the removal of GM1 from raft fractions. GM1 was not detectable in nonraft fractions after OTG solubilization, presumably due to dilution of the sphin-

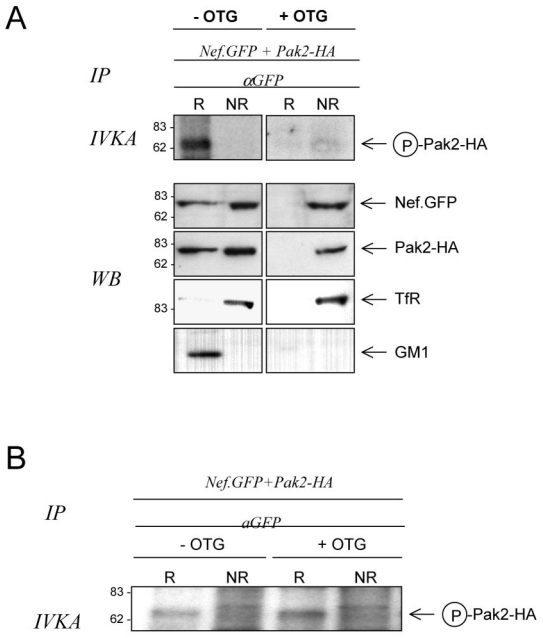

FIG. 3. Disruption of rafts interferes with Pak activation by Nef.GFP. (A) Lipid raft flotation analysis from transfected 293T cells expressing Nef.GFP and Pak2-HA. Cell lysates were split in two equal aliquots. One aliquot was incubated with 60 mM OTG (+OTG) prior to flotation analysis, whereas the other aliquot remained untreated (−OTG). After separation by Optiprep ultracentrifugation, the raft fraction (R, fraction 2) and the pooled nonraft fractions (NR, fractions 3 to 8) were analyzed. The fractions were subjected to anti-GFP immunoprecipitation and subsequent IVKA to assay their content on Nef-associated kinase activity and analyzed directly by Western blotting (WB) with the indicated antibodies for overall protein content. (B) Control IVKA for the effects of OTG on Nef-associated kinase activity. Raft (R) and nonraft (NR) fractions from 293T cells expressing Nef.GFP and Pak2-HA were left untreated (−OTG) or incubated with 60 mM OTG (+OTG) prior to anti-GFP immunoprecipitation and subsequent IVKA. Note that the faint bands in the NR fractions are nonspecific.

golipid in these fractions and the low sensitivity of the CTx-HRP Western blotting procedure. Similarly, both Nef.GFP and Pak2 proteins could not be detected in raft fractions after OTG treatment. Importantly, OTG-mediated raft disruption inhibited activation of Pak2 by Nef. Despite the presence of significant amounts of Nef.GFP and Pak2 proteins in nonraft fractions after raft disruption, Nef.GFP failed to associate with Pak2 activity. Furthermore, the association of Nef.GFP with Pak activity was also inhibited when lipid rafts were disrupted by cholesterol depletion from Jurkat cells with 10 mM methyl β-cyclodextrin for 20 min in fetal calf serum-free medium (data not shown). To control whether these effects of OTG were a nonspecific consequence of the detergent on the kinase, pooled raft and nonraft fractions of Nef.GFP-expressing 293T cells were split into two identical aliquots and then subjected to anti-GFP immunoprecipitation and subsequent IVKA directly (−OTG) or after the addition of 60 mM OTG (+OTG) (Fig. 3B). Comparable levels of Nef-associated Pak2 activity were detected in rafts under both conditions. Thus, OTG treatment per se did not affect the activity of Nef-associated Pak2. We conclude that lipid rafts are not only the preferred microenvironment for the activation of Pak by Nef but that incorporation into these domains is an essential step in the activation process.

Palmitoylation increases raft incorporation of Nef. Only a minor population of Nef was present in lipid rafts at steady state. To further corroborate our finding that raft localization correlated with the activation of Pak, we sought to experimentally enhance the incorporation of Nef into lipid rafts as a tool to further study the underlying processes. Since palmitoylation is regarded as a strong trigger for the partitioning of cytosolic proteins in the detergent-insoluble fraction (56), a palmitoylation acceptor site was created at the N terminus of Nef by the introduction of a cysteine residue at position 3 (Fig. 4A). Like the palmitoylated proteins Fyn and Lyn (55), this mutant Nef protein (PalmNef) therefore should carry both myristoylation and palmitoylation and, as a consequence, should be efficiently incorporated into lipid rafts. Palmitoylation of PalmNef.GFP, but not Nef.GFP or G2ANef.GFP, was readily detected upon labeling of Nef-expressing 293T cells with ^{3}H palmitic acid (Fig. 4B, bottom panel). Expectedly, PalmNef.GFP was incorporated markedly more efficiently into lipid rafts than Nef.GFP (Fig. 4C). This distribution was not affected by the relative low transfection efficiency of the PalmNef.GFP expression plasmid in this experiment (compare with Fig. 5B). In contrast, raft incorporation of Nef was completely abolished when the myristate at the N terminus was lacking (G2ANef.GFP). This effect of Nef palmitoylation was quantified by Western blotting of serial dilutions of the raft fractions. Equal volumes of raft and nonraft fractions were analyzed by Western blotting for their content of Nef (Fig. 4D). For Nef.GFP, bands of similar intensity were detected with the undiluted raft and the nonraft fraction at 1:4 dilution (lower panel). Since the nonraft fraction spread over fractions 7 and 8, we estimated that ca. 12.5% of the total Nef.GFP was present in lipid raft fractions. In contrast, for PalmNef.GFP, a 1:4 dilution of the raft fraction gave a comparable signal to the undiluted nonraft fraction, indicating that at least 50% of total PalmNef.GFP in the lysate fractionated with lipid rafts (upper panel). Thus, the additional palmitoylation site increased the raft association of Nef significantly. This enhanced affinity for rafts was also detected upon raft clustering in Jurkat cells, in which PalmNef.GFP was not present diffusely in the cytoplasm and partially colocalized with raft clusters at the plasma membrane (Fig. 2B). However, the amount of plasma membrane raft clusters positive for Nef was only moderately increased for PalmNef.GFP compared to Nef.GFP in most cells. This was most likely due to trapping of significant amounts of PalmNef.GFP in detergent-insoluble microdomains of intracellular membranes. We have not yet further characterized the identity of these membranes, but they might represent organelles that Nef is known to associate with in T cells, such as the *trans*-Golgi network (28), as well as structures that might only be targeted by Nef upon palmitoylation. In a minor population of cells, PalmNef.GFP was found exclusively at the plasma membrane (data not shown). We conclude that the presence of the palmitoylation acceptor site in Nef increases its raft incorporation in multiple cellular compartments and sustains the interaction of Nef with individual rafts.

Nef recruits Pak into lipid rafts, and increased raft localization of Nef augments its association with Pak activity. We next tested whether the increased raft localization of PalmNef.

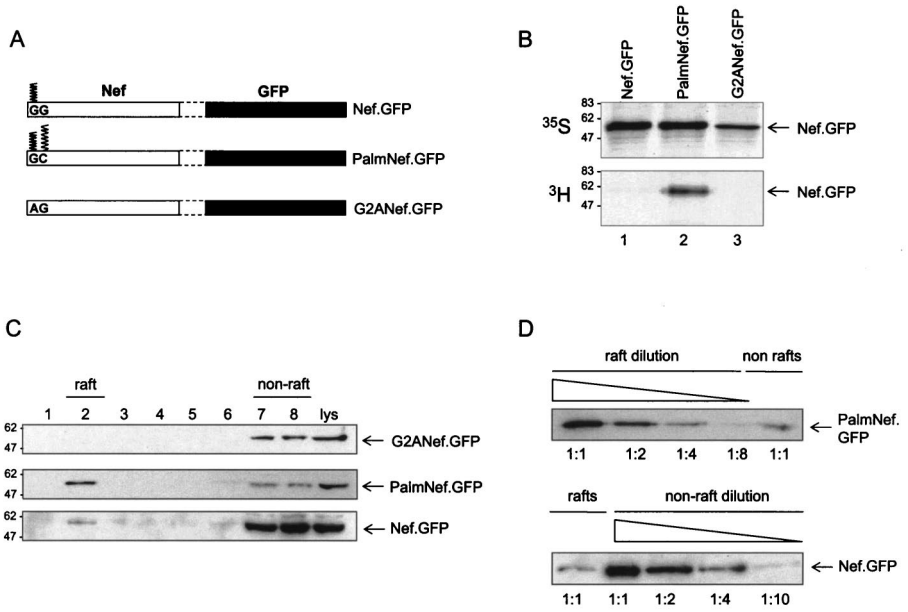

FIG. 4. Introduction of a palmitoylation acceptor site enhances raft association of Nef.GFP. (A) Schematic presentation of the Nef.GFP fusion proteins analyzed. Short and long jagged lines represent myristoyl and palmitoyl fatty acids, respectively. In PalmNef.GFP, a novel palmitoylation site was created by the G3C mutation. G2ANef.GFP cannot be myristoylated because the myristoyl acceptor glycin at position 2 was mutated to alanine. (B) Metabolic labeling of Nef.GFP fusion proteins in 293T cells. Shown are autoradiographs of anti-GFP immunoprecipitates from lysates of cells expressing the indicated Nef.GFP fusion proteins after metabolic labeling with [^{35}S]Met-[^{35}S]Cys (upper panel) or [^{3}H]palmitic acid (lower panel), respectively. Films were exposed overnight or for 6 weeks, respectively. (C) Raft flotation analysis of transfected 293T cells expressing the indicated Nef.GFP fusion proteins. Presented are Western blots with the anti-Nef antibody of equal volumes of individual gradient fractions and the unfractionated cell lysate (lys). (D) Estimation of the efficiency of raft incorporation of Nef. Shown are Western blots of serial dilutions of raft or nonraft fractions derived from 293T cells expressing PalmNef.GFP and Nef.GFP, respectively. Note that nonrafts spread over two fractions of the gradient (fractions 7 and 8), whereas rafts were concentrated in fraction 2. Samples used for quantification of protein content of nonraft fractions are therefore diluted by a factor 2 compared to raft fractions.

GFP would enhance the activity of Nef in the Pak assay and directly compared the amounts of Pak activity associated with PalmNef.GFP to those associated with Nef.GFP in Jurkat cells (Fig. 5). In these experiments, the association of Nef.GFP, but not G2ANef.GFP or GFP, with Pak activity was readily detected by the presence of the phosphorylated p62 band in the IVKA reaction after anti-GFP immunoprecipitation (Fig. 5A, left panel, IVKA). Despite similar expression levels and stability of all GFP proteins (Fig. 5A, right panel, WB), the IVKA from cells expressing PalmNef.GFP yielded consistently stronger phosphorylation of Nef-associated Pak than Nef.GFP (compare lanes 2 and 4). This increase was ~7-fold when the autophosphorylated p62 Pak signal was quantified by using a phosphorimager in the experiment shown, ranging from three- to eightfold in independent experiments. Thus, the preferential incorporation of PalmNef.GFP into lipid rafts coincided with a significant increase of Nef-associated Pak activity. To verify whether this increased association of Pak activity with PalmNef.GFP could also be detected upon raft fractionation, Nef-associated Pak2 activity was compared between raft and nonraft fractions of 293T cells expressing Nef.GFP and PalmNef.GFP (Fig. 5B). Consistent with our previous results, PalmNef.GFP was enriched in the raft fraction relative to Nef.GFP and correlated with an elevated Nef-associated kinase activity in the raft fraction. Importantly, when assayed for the distribution of Pak2 protein by Western blotting (bottom panel), Pak2 was absent in the raft fraction in GFP expressing control cells but readily detectable in rafts in the presence of Nef.GFP and PalmNef.GFP. These results demonstrate that Nef, either directly or indirectly, induces the translocation of Pak2 into lipid rafts. However, raft targeting of Pak2 was not markedly different between Nef.GFP and PalmNef.GFP, suggesting that the increased Pak activity associated with PalmNef.GFP is not a consequence of more efficient recruitment of the kinase. Based on these fractionation experiments we could not differentiate whether Nef recruits Pak to intracellular or plasma membrane rafts. We therefore analyzed the effect of Nef expression on subcellular localization of Pak2 in Jurkat cells upon raft clustering (Fig. 5C). In cells expressing G2ANef (Fig. 5C, row C), Pak2 was found mostly diffusely in the cytoplasm and partially at cellular membranes. In contrast, coexpression of Nef.GFP (Fig. 5C, row A) or PalmNef.GFP (Fig. 5C, row B) resulted in the predominant localization of Pak2 at the plasma membrane with slight but significant colocalization with Nef positive CTx clusters. Of note, Pak2 was not detected at intracellular membranes enriched with PalmNef.GFP (B). We conclude that Nef targets Pak to lipid rafts at the plasma membrane and that the relative enhancement of the raft localization of Nef results in increased levels of Nef-associated Pak activity.

Nef activates Pak2 in lipid rafts. The results described above demonstrated that lipid raft targeting of Pak is required for the

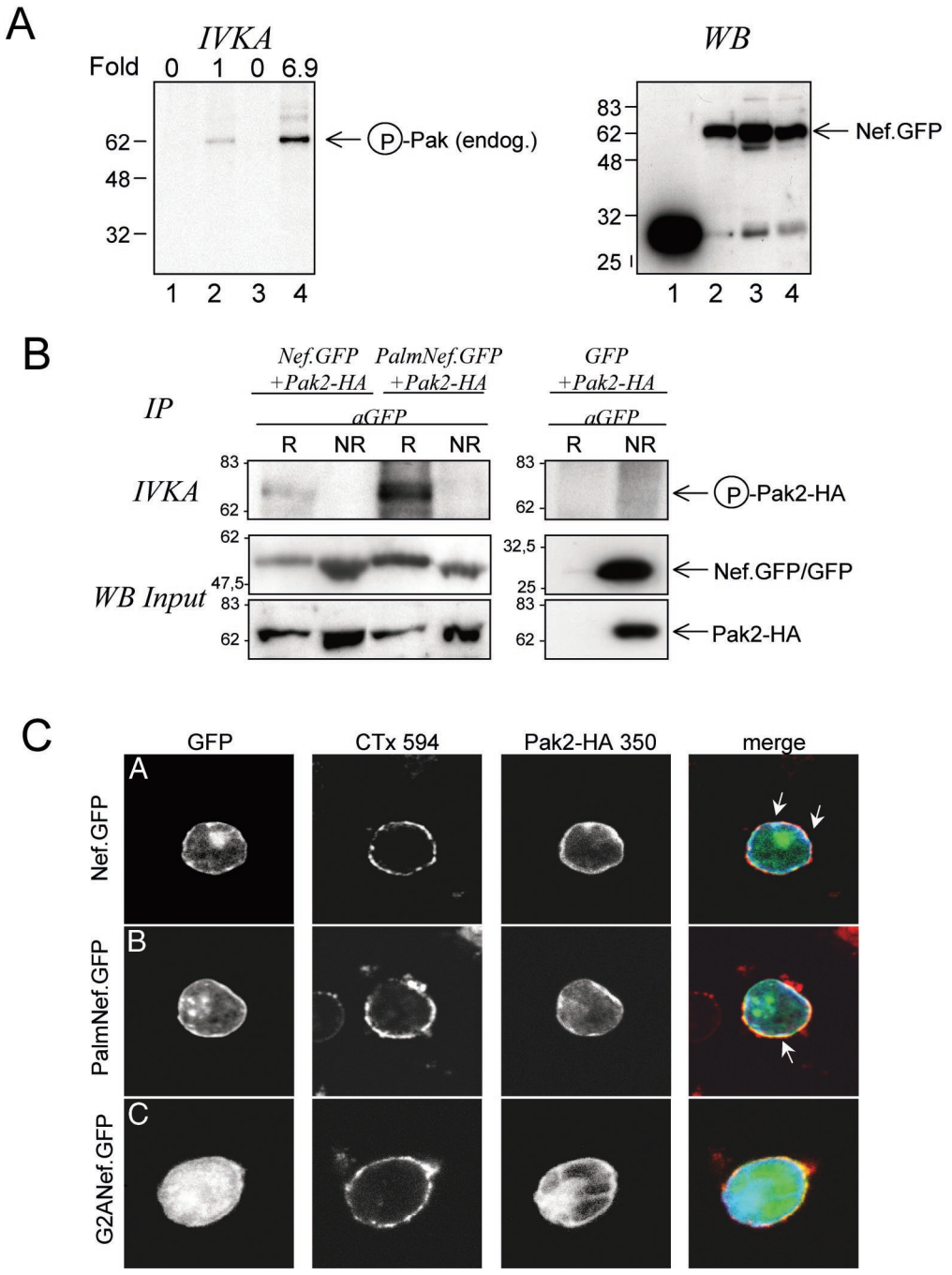

FIG. 5. Nef.GFP recruits Pak into plasma membrane lipid rafts and PalmNef.GFP associates with elevated levels of Pak activity. (A) Lane numbers indicate the GFP fusion protein expressed as follows: lanes 1, GFP; lanes 2, Nef.GFP; lanes 3, G2ANef.GFP; and lanes 4, PalmNef.GFP. IVKA, in vitro kinase reactions of anti-GFP immunoprecipitates from lysates of Jurkat cells expressing various GFP fusion proteins. Band intensities were quantified by phosphorimager analysis. The numbers at the top indicate the relative intensities of the phosphorylated p62 Pak band. WB, anti-GFP Western blot analysis of the cell lysates used for IVKA showing similar expression levels of the respective Nef.GFP derivatives. (B) Lipid raft flotation analysis from transfected 293T cells expressing Pak2-HA, together with Nef.GFP, PalmNef.GFP, or GFP, respectively. After separation by Optiprep ultracentrifugation, the raft fraction (R, fraction 2) and the pooled nonraft fractions (NR, fractions 7 and 8) were analyzed. The fractions were subjected to anti-GFP immunoprecipitation and subsequent IVKA to assay their content on Nef-associated kinase activity and then analyzed directly by Western blotting (WB) with the indicated antibodies for overall protein content. (C) Jurkat cells expressing the indicated Nef.GFP fusion proteins and Pak2-HA (rows A to C) were subjected to raft clustering by incubation with Alexa 594-conjugated CTx, cross-linking with the anti-CTx antibody, and subsequent staining with the anti-HA antibody that was detected with an Alexa 350-conjugated secondary antibody. GFP, Alexa 594, and Alexa 350 fluorescence was analyzed by confocal microscopy. The merge panel depicts the overlay of the three fluorescence channels, with arrows indicating plasma membrane clusters with colocalization of the three colors. Presented are single representative sections.

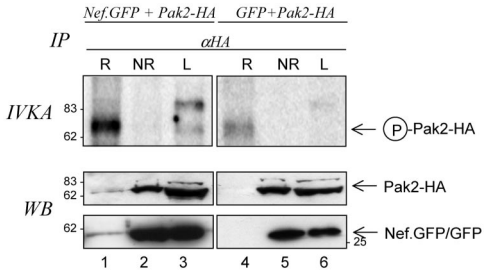

FIG. 6. Activation of Pak by Nef.GFP occurs in lipid rafts. Analysis of the raft fraction (R, fraction 2) and pooled nonraft fractions (NR, fractions 7 and 8) or the total cell lysates (L) of 293T cells expressing the indicated proteins. The upper panels (IVKA) show products from in vitro kinase reactions after immunoprecipitation with the anti-HA antibody scoring for total Pak activity. The distributions of Pak2 and Nef.GFP/GFP are shown in the Western blot (WB) analysis of the fractions in the lower panel.

detection of Nef-associated Pak activity. To test directly whether lipid raft incorporation is a prerequisite for the activation of Pak2 by Nef, we compared the overall effects of Nef on the activity of Pak2. To this end, anti-HA antibodies were used to immunoprecipitate Pak directly from the lysates and gradient fractions of 293T cells, and the kinase activity in these immunoprecipitates was analyzed (Fig. 6). As expected, Pak2 activity was barely detectable in the total lysate and weak in the raft fraction of GFP-expressing control cells (IVKA, lanes 4 to 6), and no Pak2 protein was detected in lipid rafts by Western blotting (WB, lanes 4 to 6). In contrast, the presence of Nef resulted in a slight but significant recruitment of Pak2 into the raft fraction (WB, lanes 1 to 3), coinciding with a marked increase in Pak2 activity in the total lysate and in particular in the raft fractions (IVKA, lanes 1 to 3). Thus, the presence of Nef in cells leads to overall Pak2 activation, and this appears to be largely due to lipid raft recruitment of Pak2 by Nef. In addition to autophosphorylation of Pak2, phosphorylation of an unidentified protein with an apparent molecular mass of ca. 85 kDa was also induced in the presence of Nef. In contrast to autophosphorylated Pak2, phosphorylation of this 85-kDa protein was not observed in the raft fraction. This suggests that the phosphorylation of the 85-kDa substrate involves raft and nonraft components that are separated upon raft flotation.

Activation of Pak2 by Cdc42 and Rac occurs in lipid rafts. Our results indicated that Nef activates Pak via the recruitment of the kinase into plasma membrane rafts. Since physiological activation of Pak involves its recruitment to the plasma membrane (17), we wanted to explore whether lipid rafts are also involved in the activation of Pak2 by its cellular upstream regulators Cdc42 and Rac (Fig. 7A). 293T cells transiently expressing Pak2 in the presence of constitutive active Cdc42 (caCdc42; lanes 1 to 3), constitutive active Rac (caRac; lanes 4 to 6), or a GFP control (lanes 7 to 9) were subjected to raft flotation analysis, and the autophosphorylation activity of Pak2 was assayed from pooled raft and nonraft fractions (Fig. 7, R and NR, respectively), as well as the total cell lysate (L), after anti-HA immunoprecipitation. As expected, coexpression of caCdc42 and caRac, together with Pak2, caused strong activation and autophosphorylation of the kinase (IVKA, compare lanes 3 and 6 to lane 9). This autophosphorylation almost exclusively occurred in the raft fractions and not in the nonraft fractions (IVKA, compare lanes 1 and 2 to lanes 4 and 5) and was markedly enriched in rafts in the presence of caCdc42 (IVKA, compare lanes 1 and 3). Importantly, Pak2 protein was strongly recruited to lipid raft fractions in the presence of caCdc42 and caRac (WB, compare lanes 1 and 4 to lane 7). Confocal microscopy of transfected Jurkat cells (Fig. 7C) revealed that this effect was paralleled by a strong recruitment of Pak2 to the plasma membrane and not to intracellular structures upon coexpression of caRac (row B) or caCdc42 (row C) compared to cells coexpressing GFP as a control (row A). In those cells, significant colocalization of the active GTPases, Pak2, and clustered CTx was observed. Thus, activation of Pak2 by caCdc42 and caRac coincides with targeting of the kinase to plasma membrane rafts. As observed with Nef, activation by the GTPases resulted in the autophosphorylation of Pak2, as well as the appearance of an additional phosphorylated protein of ~85 kDa, which, however, was not strongly enriched in rafts (Fig. 7A). We conclude that, similar to the scenario with Nef, lipid rafts represent a preferential microenvironment for the induction of Pak2 autophosphorylation by its physiological activators Rac and Cdc42. However, the increase of Pak2 autophosphorylation, as well as phosphorylation of the 85-kDa protein, was more pronounced in the presence of constitutive active GTPases than in the presence of Nef.GFP (compare Fig. 6 and 7A). Together, whereas both Nef and the GTPases recruit active Pak2 into lipid rafts, Nef is a weaker activator of Pak2 than caRac and/or Cdc42.

To confirm the importance of raft incorporation for the activation of Pak2 by the GTPases, we tested whether raft disruption also interfered with the activation of Pak2 by caCdc42 and caRac (Fig. 7B). Again, autophosphorylation of Pak2 induced by both active GTPases showed a strong preference for lipid rafts. This robust activation was strongly diminished upon disruption of rafts by OTG, a loss of kinase activity that was paralleled by a lack of Pak2 recruitment into lipid rafts in the presence of OTG. As for the Nef-associated Pak activity, OTG had no effect of Pak2 activity upon induction by constitutive active GTPases (data not shown). The correlation between raft recruitment and activity of Pak2 is also emphasized by the residual Cdc42-mediated Pak2 activity in the raft fraction that results from the incomplete solubilization of lipid rafts in this experiment. In contrast, the substantial amounts of Pak2 present in nonrafts did not display significant autophosphorylation activity. Together, these data demonstrate that activation of Pak2 by the small GTPases Cdc42 and Rac involves recruitment of the kinase into plasma membrane rafts. Thus, albeit less efficient than Cdc42 or Rac, HIV-1 Nef mimics a cellular mechanism for the induction of the Pak signaling cascade.

DISCUSSION

This study presents several lines of evidence for a role of lipids rafts in HIV-1 Nef-mediated activation of the Pak kinase. First, Nef-associated kinase activity is detected exclusively in lipid raft fractions. Second, disruption of lipid rafts prevents the association of Pak activity with Nef and, third, enhancement of raft association of Nef via an additional palmitoylation augments its ability to activate Pak. The data presented here also demonstrate that the specific localization of

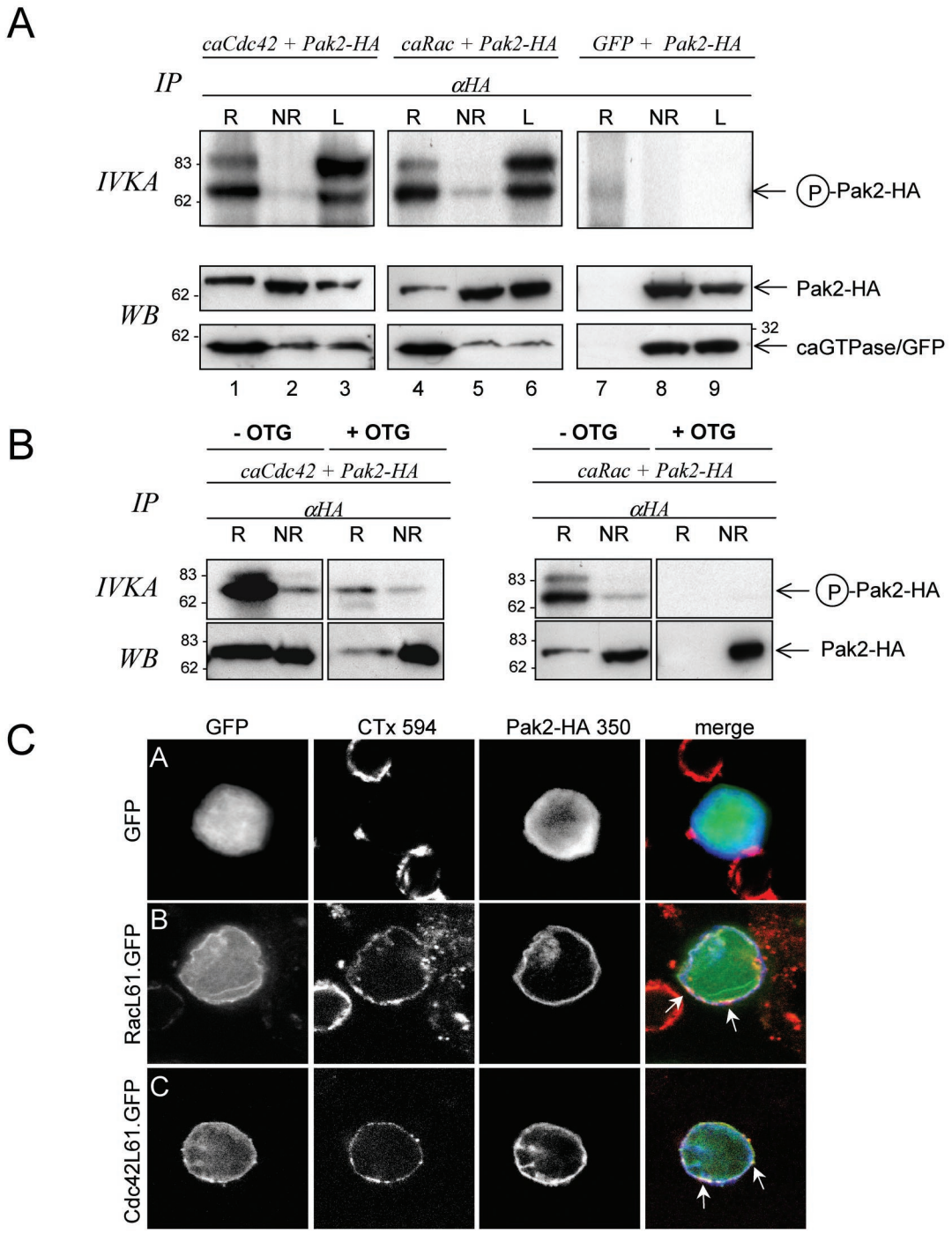

FIG. 7. Activation of Pak by Cdc42 and Rac occurs in lipid rafts. (A) Analysis of the raft fraction (R, fraction 2) and pooled nonraft fractions (NR, fractions 7 and 8) or the total cell lysates (L) of 293T cells expressing the indicated proteins. The upper panels (IVKA) shows products from in vitro kinase reactions following immunoprecipitation with the anti-HA antibody scoring for total Pak activity. The distribution of Pak2 and the GTPases or GFP are shown in the Western blot (WB) analysis of the fractions in the lower panels. (B) Effect of raft disruption on Pak2 activation by caCdc42 and caRac. Cell lysates were split in two aliquots. One aliquot was incubated with 60 mM OTG (+OTG) prior to flotation analysis, whereas the other aliquot remained untreated (−OTG). After separation by Optiprep ultracentrifugation, the raft fraction (R, fraction 2) and the pooled nonraft fractions (NR, fractions 7 and 8) were analyzed for total Pak2 activity after anti-HA immunoprecipitation (IVKA). The fractions were also analyzed directly for protein content by Western blotting (WB) with the indicated antibodies. (C) Jurkat cells expressing the indicated GFP fusion proteins and Pak2-HA (rows A to C) were subjected to raft clustering by incubation with Alexa 594-conjugated CTx, cross-linking with the anti-CTx antibody, and subsequent staining with the anti-HA antibody that was detected with an Alexa 350-conjugated secondary antibody. GFP, Alexa 594, and Alexa 350 fluorescence was analyzed by confocal microscopy. The merge panel depicts the overlay of the three fluorescence channels, with arrows indicating plasma membrane clusters with colocalization of the three colors. Presented are single representative sections.

Pak activation to lipid rafts at the plasma membrane involves the physical recruitment of the kinase that is otherwise excluded from rafts. Importantly, activation of Pak2 by its physiological activators Cdc42 and Rac also occurred in plasma membrane lipid rafts, required raft integrity, and coincided with the recruitment of Pak2 into detergent-insoluble microdomains. Together, these data suggest that Nef mediated recruitment of Pak is essential for the subsequent activation of the kinase and indicate that the viral protein mimics a cellular strategy for Pak activation.

Only small portions of total cellular Nef and Pak proteins are present in lipid rafts at steady state, and yet these subpopulations are apparently sufficient to produce the robust Nef-associated Pak activity. These findings are in line with a number of reports that conclude that the Nef-Pak interaction is transient and difficult to detect by Western blotting (3, 22, 48, 53, 61). Increasing the local protein concentrations by lipid raft incorporation may therefore be of particular benefit for the efficiency of Pak activation by Nef. In addition, this mechanism will ensure that activation only occurs locally, preventing random hyperactivation of Pak by the large amounts of Nef protein present in infected cells. Despite sharing the mechanism of raft recruitment for activation of Pak, Nef appears to be a weaker inductor than Cdc42 and Rac. This likely reflects that the constitutive active GTPases very efficiently and directly recruit Pak, thereby inducing the kinase without further cofactors. In contrast, Nef recruits Pak as part of a signalosome, including Vav and Rac/Cdc42, with which activation of Vav is thought to trigger GTPase activity (23). Conceivably, such an indirect mechanism might result in less-pronounced net activation of the effector Pak than triggering the kinase directly via its upstream activating GTPase. Such a model predicts an enhancement of GTPase activity by Nef, which would explain the abolition of Pak activation by Nef by dominant-negative Rac and Cdc42 (41). Of note, the experiments with PalmNef.GFP suggested that the amounts of Pak recruited by Nef are not limiting for the activation of the kinase. The enhanced association of PalmNef.GFP with Pak activity therefore might reflect increased stability of the Nef-Pak complex upon improved raft targeting rather than an elevated ability to recruit the kinase.

The effects of Nef on total Pak activity described here are stronger than those reported by Renkema et al., who coexpressed active GTPases to detect Nef-associated Pak activity (54). In contrast, the effects described here are less pronounced than the massive activation of Pak by Nef observed by Arora et al. (3). Given the involvement of the GTPases in the activation by Nef and the dependence of endogenous GTPase activity levels on the specific cell culture conditions, it can be hypothesized that the intrinsic levels of GTPase activity in the different cell lines cultured at various conditions in these studies could account for these differences. When GTPase activities are very low, additional activation may be required to trigger association with Nef, while high intrinsic GTPase levels may result in the association of elevated Pak activity levels with Nef.

A role of lipid rafts in Pak activation fits well with the overall model that plasma membrane targeting of Pak in cells causes its activation (4, 15). However, our findings suggest that the specific microenvironment of lipid rafts rather than plasma membrane attachment per se is required to trigger activation. Our data allow us to conclude that GTPase-driven activation of Pak occurs in lipid rafts. These results are in line with the previous finding that activation of Pak via membrane targeting requires GTPase activity (40). Similarly, activation of Pak via cell adhesion involves plasma membrane recruitment of Pak in a Cdc42-dependent fashion (17). Based on our results, it can be envisioned that activation by Rac and Cdc42 is achieved through the recruitment of Pak into lipid rafts in which the kinase is activated by the GTPase. However, we cannot exclude that Pak is activated shortly before raft incorporation and that the results presented here reflect the significantly increased affinity of activated Pak to lipid rafts. Since lipids have been implicated in the regulation of Pak activity after the initial trigger by the GTPase (4, 15), it is tempting to speculate that the particular lipid composition of rafts serves to increase Pak activity. In both scenarios, Pak would reach full activity only upon incorporation in lipid rafts. The exact mechanism of raft recruitment and concomitant activation of Pak by Nef and the GTPases warrants further investigation.

Activation of the TCR cascade by Nef results in the induction of a gene array that is 97% identical to that triggered by stimulation with an anti-CD3 antibody (65). Our data suggest that this stunning overlap can be attributed in part to the fact that Nef acts as an endogenous TCR stimulus that, analogous to exogenous activation, recruits the TCR machinery into lipid rafts. Despite these similarities, activation of unstimulated primary human T cells via anti-CD3 induces an activation state that allows HIV to replicate, whereas Nef alone only induces a semipermissive state (21). Thus, subtle differences between both stimuli, possibly reflected in the differential activation of the remaining 3% of target genes, should govern important functional consequences. Based on our results, we propose that altered composition and activity of the signalosomes recruited into lipid rafts by Nef and anti-CD3 activation will account for this difference. It will therefore be of great interest to use Nef as a tool for the characterization of this intermediate activation state in the future. Importantly, evolutionary pressure appears to prevent emergence of Nef alleles with augmented signaling capacities. Creating a more efficient signaling adaptor, i.e., by introducing a palmitoylation acceptor as in the present study, would only require mutation of a single codon in the *nef* gene. However, none of the known *nef* sequences encode a cysteine at position 3, indicating strong counterselection against this modification in vivo. The fatal consequences of elevated signaling capacities by Nef in general are evidenced by the highly aggressive SIV strain pbj14, which causes an acutely lethal infection in vivo, thereby limiting its own transmission (18). This increased pathogenicity was attributed to the creation of an additional ITAM in Nef that results in strong activation of TCR signaling causing massive virus spread in resting T cells and oncogenicity (18, 43). Therefore, the activation level induced by Nef appears to be closely balanced with the consequences of enhanced T-cell activation to ensure optimal viral replication in vivo.

Thus, the present study proposes raft recruitment as an essential step of Pak activation by Rac and Cdc42, as well as by the HIV pathogenicity factor Nef. Since enhancement of virion infectivity also requires raft incorporation of Nef (76), detergent-insoluble microdomains emerge as a central platform for

Nef function at the plasma membrane. Future studies will address the involvement of lipid rafts in other activities of Nef.

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