Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1SF2 Nef

Simone I. Giese, Ilka Woerz, Stefanie Homann, Nadine Tibroni, Matthias Geyer, Oliver T. Fackler

a Department of Virology, University of Heidelberg, 69120 Heidelberg, Germany
b Max Planck Institute for Molecular Physiology, Department of Physical Biochemistry, 44227 Dortmund, Germany

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Abstract

Membrane association is believed to be a prerequisite for the biological activity of the HIV-1 pathogenicity factor Nef. Attachment to cellular membranes as well as incorporation into detergent-insoluble microdomains (lipid rafts) require the N-terminal myristoylation of Nef. However, this modification is not sufficient for sustained membrane association and a specific raft-targeting signal for Nef has not yet been identified. Using live cell confocal microscopy and membrane fractionation analyses, we found that the N-terminal anchor domain (aa 1–61) is necessary and sufficient for efficient membrane binding of Nef from HIV-1SF2. Within this domain, highly conserved lysine and arginine residues significantly contributed to Nef’s membrane association and localization. Plasma membrane localization of Nef was also governed by an additional membrane-targeting motif between residues 40 and 61. Importantly, two lysines at positions 4 and 7 were not essential for the overall membrane association but critically contributed to Nef’s incorporation into lipid raft domains. Cell surface receptor downmodulation was largely unaffected by mutations of all N-terminal basic residues, while the association of Nef with Pak2 kinase activity and its ability to augment virion infectivity correlated with its lysine-mediated raft incorporation. In contrast, all basic residues were required for efficient HIV-1 replication in primary human T lymphocytes but did not contribute to the incorporation of Nef into HIV-1 virions. Together, these results unravel that Nef’s membrane association is governed by a complex pattern of signature motifs that differentially contribute to individual Nef activities. The identification of a critical raft targeting determinant and the functional characterization of a membrane-bound, non-raft-associated Nef variant indicate raft incorporation as a regulatory mechanism that determines the biological activity of distinct subpopulations of Nef in HIV-infected cells.

Keywords: HIV Nef; Membrane association; Raft incorporation; Virion incorporation; Cell surface receptor modulation; Virus infectivity and replication

Introduction

The Nef protein encoded by the primate lentiviruses HIV and SIV augments virus replication in vivo and thus increases the pathogenic potential of these viruses. This impact on the development of infection-associated disease most likely reflects combinatorial effects of Nef on target host cells that synergistically optimize virus replication. These activities include immune evasion mechanisms leading to escape of recognition of infected cells by cytotoxic T-cells (Collins et al., 1998; Schwartz et al., 1996; Xu et al., 1999), decreased MHC-II restricted antigen presentation and prevention of IgG class switching (Qiao et al., 2006; Stumptner-Cuvelette et al., 2001, 2003). Nef also impacts directly on the intrinsic replicative potential of these viruses by, e.g., augmenting virion infectivity, extending the life span of infected cells, preventing superinfection and sensitizing T lymphocytes for activation and thus HIV replication (Aiken and Trono, 1995; Benson et al., 1993; Fortin et al., 2004; Keppler et al., 2006; Michel et al., 2005; Schwartz et al., 1995; Wolf et al., 2001). Nef exerts this multitude of effects by interfering with vesicular sorting and signal transduction processes as well as via its ability to act as an adaptor molecule for select cellular pathways (Arora et al., 2002; Geyer et al., 2001; Tolstrup et al., 2004).

Although Nef exerts an astonishingly high number of activities via a complex array of distinct molecular mechanisms,
two common principles appear to underlay its biological activity: First, Nef affects cellular machines by interaction with target proteins. As expected from its pleiotropic effector functions, the dynamic regulation of Nef’s activities involves a steadily increasing number of cellular interaction partners (Geyer et al., 2001; Saksel, 2004; Tolstrup et al., 2004). Second, its association with cellular membranes is believed to be essential for virtually all of its effects in infected cells. Based on membrane fractionation analyses, a significant fraction of Nef is associated with cellular membranes but Nef is also distributed in the cytosol without apparent membrane association (Bentham et al., 2006; Coates et al., 1997; Fackler et al., 1997; Kaminchik et al., 1994; Niederman et al., 1993). Confocal microscopy suggested that most of the membrane-bound Nef protein associates with yet undefined intracellular membranous organelles while a minor fraction of Nef is localized at the inner leaflet of the plasma membrane (Bentham et al., 2006; Craig et al., 2000; Fackler et al., 2006; Greenberg et al., 1997; Keppler et al., 2005). While it is unclear what determines the association of Nef with select membrane compartments, Nef’s overall membrane attachment is primarily governed by its N-terminal myristoyl moiety. Consequently, Nef mutants that lack the N-terminal myristoylation because of mutations of the myristoyl-acceptor glycine at position 2 display at least significantly reduced biological activity in most assays for Nef function (reviewed in Geyer et al., 2001). However, studies on other cytosolic proteins that are tethered to cellular membranes revealed that myristoylation alone is insufficient to provide sustained membrane association and several additional signals that increase membrane affinity and sometimes provide specificity for select membrane compartments within cells were identified (Resh, 1999, 2004). These “second signals” include additional fatty acid modifications such as palmitoylation in the case of Src family kinases (e.g., Fyn, Yes and Lck) or protein interaction sites as with the interaction of caveolin with GoI (Galbiati et al., 1999; McCabe and Berthiaume, 1999; McCabe and Berthiaume, 2001; Resh, 1999). Alternatively, a cluster of basic amino acids downstream of the myristoylated N-terminus facilitates membrane association of, e.g., kinases of the Src family or the HIV-1 Gag protein (Resh, 1999, 2004). Consistent with this scenario, myristoylation of Nef was recently shown not to be the only determinant for Nef’s attachment to cell membranes and for its incorporation into HIV-1 particles (Bentham et al., 2006; Fackler et al., 2006). Rather, combinatorial membrane targeting via the myristoyl moiety and basic residues, together also referred to as SH4 domain, has been proposed for the Nef protein of HIV-1NL4-3 (Welker et al., 1998). In this case, lysine as well as arginine residues in the Nef N-terminus contribute to its membrane binding and virion incorporation, as well as to effects on virus replication and cell surface CD4 exposure (Bentham et al., 2006; Welker et al., 1998). In contrast, we recently found that for Nef from HIV-1SF2, N-terminal arginine residues are dispensable for its biological activity in HIV-1-infected T-lymphocytes (Fackler et al., 2006), suggesting the existence of further signature motifs that determine Nef’s membrane association.

In addition to its association with select cell membranes, a subpopulation encompassing approximately 5% of total Nef protein is incorporated into detergent-resistant microdomains (lipid rafts) (Alexander et al., 2004; Krautkramer et al., 2004; Sol-Foulon et al., 2004; Wang et al., 2000; Zheng et al., 2001). While this association also critically depends on Nef’s myristoylation, specific determinants for raft incorporation have not been identified to date. The lack of such specificity significantly complicated investigations on the biological relevance of the raft-associated Nef population. Using Nef variants that were artificially enriched in lipid raft domains due to an additional palmitoylation site, we reported that raft incorporation of Nef is essential for its ability to associate with Pak2 kinase activity (Krautkramer et al., 2004). In contrast, effects of Nef on modulation of surface receptors such as CD4 and MHC-I as well as enhancement of virion infectivity were shown to be independent of lipid rafts by using an identical experimental approach (Sol-Foulon et al., 2004).

In this present study, we set out to define the membrane binding determinants of Nef from HIV-1SF2 using life cell confocal microscopy and membrane fractionation analyses. We report that the N-terminal anchor domain of Nef is sufficient for efficient membrane attachment and that an N-terminal basic cluster consisting of lysine and arginine residues contributes to membrane association of Nef. However, this support of membrane binding was dispensable for Nef’s biological activity in receptor modulation and virion incorporation. In contrast, the lysine residues were specifically required for raft incorporation of Nef and essential for Nef’s association with Pak2 activity, enhancement of virion infectivity and virus replication in primary human T lymphocytes. Together, these results establish that select Nef activities require differential membrane binding avidities, characterize a specific raft incorporation determinant in Nef and shed further light on the role of lipid rafts in the biological activity of the HIV pathogenicity factor.

Results

The N-terminal anchor domain is necessary and sufficient for membrane association of HIV-1SF2 Nef

The overall three-dimensional organization of the HIV-1 Nef protein is governed by three distinct structural entities: the largely unstructured N-terminal anchor domain (aa 1–61 in the case of Nef from HIV-1SF2), a well folded core domain (aa 62–210) and a C-terminal flexible loop (aa 152–184) (reviewed in Geyer et al., 2001; Geyer and Peterlin, 2001). Efficient membrane association of Nef is thought to determine its functionality and to depend on its N-terminal myristoylation. However, myristoylation alone provides insufficient avidity for sustained membrane association in cells and Nef must therefore employ additional targeting motifs for membrane anchorage (Resh, 1999, 2004). A stretch of arginine residues near the Nef N-terminus is thought to participate in mediating membrane association of Nef from HIV-1NL4-3 (Bentham et al., 2006; Welker et al., 1998). We recently described that these arginine residues are not essential for the biological activity of Nef from
HIV-1SF2 in infected T-lymphocytes (Fackler et al., 2006). We therefore set out to identify the membrane binding determinants of Nef from HIV-1SF2. This allele is widely used as a prototypic HIV-1 Nef variant due to its high biological activity in all assay systems analyzed thus far (Arora et al., 2000; Baur et al., 1994; Fackler et al., 2001; Keppeler et al., 2005; Krautkramer et al., 2004; Renkema et al., 1999).

In order to directly compare the subcellular localization of Nef in living cells with its membrane association determined by biochemical approaches, we generated a series of expression constructs for Nef proteins fused to GFP (Nef.GFP) (Fig. 1). In line with a recent report (Bentham et al., 2006), metabolic labeling experiments with tritium-labeled myristic acid did not reveal significant differences in the relative degree of myristoylation between these constructs in human cells (data not shown). We first attempted to narrow down the region of Nef that bears the major membrane binding determinants by using various Nef fragments fused to GFP. These included the anchor (henceforth referred to as 1–61) and core (62–210, carrying an additional myristoylation signal) domains of Nef as well as individual fragments of its N-terminal anchor (1–29, 1–40, 1–50). Full-length SF2 Nef (wt) and its non-myristoylated variant (G2A) served as references. Live cell imaging of transfected HeLa cells was employed to avoid the artificial reduction of apparent membrane association of SH4 domain containing proteins observed upon cell fixation (McCabe and Berthiaume, 1999) (Fig. 2A). Confocal microscopy of wt, expectedly, revealed its association with membranous organelles, vesicular structures in the cytoplasm as well as a diffuse cytosolic distribution. Importantly, this real-time analysis also revealed a prominent association of Nef with the plasma membrane that was slightly more apparent than in fixed samples (data not shown). In line with the proposed role of myristoylation in Nef’s membrane attachment, G2A was found not to be enriched at cellular membranes and was rather distributed homogeneously throughout the cell. This distribution was virtually indistinguishable from that of GFP alone, suggesting that the presence of G2A in the nucleus reflects the karyophilic properties of the GFP moiety of the fusion protein. A similar pattern was also observed for 62–210, demonstrating that the core domain of Nef does not significantly contribute to its membrane attachment and that myristoylation alone is insufficient for membrane targeting of the core domain. In contrast, the anchor domain 1–61 efficiently targeted GFP to cellular membranes with a strong specificity for the plasma membrane while only a weak diffuse cytoplasmic distribution was observed. This pronounced plasma membrane localization was also observed with the fragments 1–50 and 1–40, although membrane association appeared increasingly less prominent with decreasing length of the Nef fragment analyzed. The gradual loss of membrane association was most apparent with fragment 1–29, which was evenly distributed throughout the cell including some localization at the plasma membrane. Together, the N-terminal anchor of Nef contains all signals for

![Fig. 1. Schematic representation of Nef.GFP expression constructs used in this study. Depicted at the top is Nef.GFP from HIV-1SF2 (wt) with the structural domains and amino acids targeted by mutagenesis in this study: myristoylation-acceptor glycine at the N-terminus (depicted by the fanfold tail), two basic clusters (one consisting of K4 and K7, one comprised of R17, 19, 21 and 22), M10, W5 and W13, and the alpha helix within the anchor domain of Nef (aa 38–45: SRDLEKHG). Numbers indicate amino acid positions in Nef.](image-url)
Fig. 2. Subcellular localization of different Nef.GFP fragments in transfected HeLa cells. (A) Live cell imaging of HeLa cells transiently expressing the indicated Nef.GFP proteins. 24 h post-transfection cells were analyzed by confocal microscopy. Individual representative z-sections are presented. Scale bar=10 μm. (B) Membrane fractionation of HeLa cells analyzed in panel A. 24 h post-transfection, postnuclear extracts were fractionated into soluble (S) and pellet (P) fraction and analyzed by Western blotting for the distribution of Nef.GFP. (IN) designates unfractionated cell lysates as input control. Note that the electrophoretic mobility of various Nef.GFP fragments differs significantly due to their size differences. Transferrin receptor (TfR) and 14-3-3 proteins were analyzed as markers for S and P fractions, respectively. (C) Quantification of the membrane fractionation analysis presented in panel B. Shown is the relative distribution of the various Nef.GFP proteins into S and P fractions with the total signal from both fractions set to 100%. Data represent average values from at least three independent experiments with the indicated standard error of the mean. The statistical significance of the differences in the relative distribution to the P fraction for the indicated Nef mutants relative to the wt Nef control was analyzed by Student’s t test (***P<0.0005; **P<0.005; *P<0.05).
efficient association with the plasma membrane while the core domain bears determinants for its recruitment to intracellular membranous organelles.

Within the anchor domain, our observations suggested that the stretch between residues 29 and 61 contains a determinant for plasma membrane targeting of Nef that synergizes with its N-terminal myristoylation. We next compared these results with the distribution of the various Nef.GFP proteins upon biochemical fractionation (Fig. 2B). Postnuclear extracts of transfected HeLa cells were subjected to ultracentrifugation and separated into a soluble fraction containing all cytosolic components (S) and a pellet fraction that bears membranes, cytoskeleton and protein aggregates (P) (Niederman et al., 1993; Tritel and Resh, 2000). The cytoplasmic 14-3-3 protein and the transmembrane transferrin receptor (TIR) were used as quality control for S and P, respectively. While consistently no contamination of the P fraction by soluble material was detected, minor contaminations of the S fraction with TIR were occasionally observed. Consistent with previous reports on non-fusion Nef proteins in transfected HeLa cells or HIV-1-infected Jurkat T lymphocytes (Bentham et al., 2006; Fackler et al., 2006) and thus validating the use of GFP fusion proteins for this analysis, wt Nef.GFP was found to 30% in the pellet fraction and this was significantly reduced, but not fully abrogated, for the non-myristoylated G2A variant (10% in P) (Fig. 2C). Consistent with our imaging analysis, 62–210 was detected in P with an efficiency comparable to G2A and 1–61 was strongly enriched in the membrane fraction (73%). The shorter anchor fragments segregated in the P fraction with intermediate efficiency (50–57%). Of note, the relative amounts of 1–29 in P were comparable to those of 1–40 and 1–50 despite the lack of a pronounced plasma membrane localization of the short fragment, suggesting that 1–29 was predominantly targeted to intracellular membranes or included in protein aggregates. Together, the results of the imaging and fractionation analyses roughly correlated and emphasize the existence of a determinant for plasma membrane targeting signal in Nef between residues 29 and 61.

**Basic residues in the anchor domain synergistically contribute to Nef’s membrane association**

We next analyzed the involvement of N-terminal residues with putative membrane affinity in the membrane attachment of Nef. First, the basic amino acids previously implied in membrane binding of Nef from HIV-14,3 were mutated to alanine resulting in the KK and R4 Nef variants, carrying the (K4/7A) and (R17/19/21/22A) mutations, respectively. A combinatorial KR mutant with all lysine and arginine residues mutated to alanine was also generated. Since methionine and tryptophane residues can also mediate interactions at protein-membrane interfaces (Wimley and White, 1996), W5 and W13 were targeted by alanine mutagenesis either alone to yield the WW mutant or in combination with M10 (WMW). Finally, a combinatorial Nef variant of the WMW and R4 mutants (WR) and a deletion mutant lacking a putative amphipathic helix that serves as the interaction site for NACK and AP-1/MHC-I complexes (Δ12–39) (Baur et al., 1997; Roeth et al., 2004) were generated. All these Nef.GFP proteins were expressed to comparable levels, suggesting that the introduced mutations did not significantly affect protein stability (see Fig. 3B). Notably, mutations of the lysine residues slightly accelerated the electrophoretic mobility of Nef.GFP. Real-time confocal microscopy revealed that all these mutations slightly decreased the apparent association of Nef.GFP with the plasma membrane and increased the diffuse cytosolic distribution (Fig. 3A). These effects were most pronounced for Nef variants lacking the stretch of arginines (R4, KR and Δ12–39). In contrast, mutations in the W and K residues had less influence on Nef’s subcellular distribution. Surprisingly, despite mutation of the arginines, WR displayed a subcellular distribution that was comparable to that of wt, suggesting that the additional mutation of the tryptophanes somehow compensated for the lack of the arginines. Nevertheless, these results demonstrate that the arginine residues in the N-terminus of Nef SF2 play a significant role for its localization in living cells. Comparable results were obtained with the biochemical membrane fractionation analysis (Figs. 3B and C). Mutations of the tryptophane or lysine residues did not significantly affect the distribution between soluble and pellet fractions and the loss of the positive charges provided by the arginine stretch caused a moderate, but significant reduction in the association of Nef with the membrane pellet. To illustrate the inter-experimental variability in the distribution of Nef to the P fraction, two experiments are presented for KK that represent the minimal and maximal pellet association observed. In contrast, the KR variant almost completely failed to associate with the pellet fraction and was consistently found to be even more enriched in the cytosolic fraction than G2A. The fact that Δ12–39 retained considerable membrane affinity in the biochemical assay and plasma membrane localization in living cells together with our previous results with the N-terminal Nef fragments suggested that residues 40–61 contain an additional determinant for plasma membrane localization of Nef. This stretch contains a series of charged residues that form a short alpha helix (Geyer et al., 1999). In order to define the targeting motif involved, a series of deletions and point mutations of positively or negatively charged residues was generated, however, poor stability of all resulting Nef.GFP fusion proteins precluded further analysis (data not shown). Together, these analyses demonstrate that basic residues in the N-terminus of Nef significantly contribute to its membrane affinity.

**N-terminal lysines are critical for lipid raft incorporation of Nef**

Besides myristoylation, specific determinants that govern Nef’s incorporation into lipid rafts have not yet been identified. We addressed this question with our combined imaging and biochemical fractionation approach. As described before (Krautkramer et al., 2004), lipid rafts were visualized in transfected Jurkat T-cells via clustering of fluorescently labeled cholera toxin (CTx) (Fig. 4A) and these results were compared to those from raft flotation analysis after Triton X-100 based
Fig. 3. Subcellular localization of different Nef.GFP mutants in transfected HeLa cells. (A) Live cell imaging of HeLa transiently expressing the indicated Nef.GFP proteins. Scale bar=10 μm. (B) Membrane fractionation of HeLa cells analyzed in panel A. The relative distribution of the Nef variants in the P fraction was quantified from the blots presented to wt: 27%, G2A: 2%, WW: 41%, WMW: 39%, WR: 21%, Δ12–39: 22% (upper panel) and wt: 28%, G2A: 23%, KK: 11%, R4: 25%, KR: 5%, KK: 35% (lower panel). (C) Quantification of at least three independent membrane fractionation analyses. All analyses were performed as described in the legend to Fig. 2. The statistical significance of the differences in the relative distribution to the P fraction for the indicated Nef mutants relative to the wt Nef control was analyzed by Student’s t test (***P<0.0005; **P<0.005; *P<0.05).
Fig. 4. Raft localization of different Nef.GFP constructs. (A) Clustering of Nef.GFP with lipid rafts. 24 h post-transfection, Jurkat T-cells transiently expressing the indicated Nef.GFP proteins were subjected to raft clustering by incubation with Cholera Toxin (CTx) conjugated with Alexa-594 (KK) or Alexa-555 (all others) fluorescent dyes, respectively, and subsequently crosslinked with anti-CTx antibody. Cells were analyzed by confocal microscopy and single representative sections are presented. The merge panel depicts the overlay of both fluorescence channels. (B) Lipid raft flotation analysis from transfected 293T cells expressing different Nef.GFP fusion proteins. 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. The detergent-resistant membrane fraction (DRM, fraction 2) and the pooled non-raft fractions (NR, fractions 7 and 8) were analyzed together with the unfractionated cell lysate (L) by Western blotting for the distribution of Nef.GFP. TfR and caveolin were analyzed as markers for NR and DRM fractions, respectively.
lysis at 4 °C of transfected 293T cells (Fig. 4B). Enrichment of caveolin in the detergent-resistant membrane (DRM) fraction and of TfR in the non-raft (NR) fractions served as control for the raft flotation procedure. Expectedly, wt was found to partially co-localize with CTx-positive raft clusters at the plasma membrane and to be present in DRM fractions in a myristoylation-dependent manner (compare wt and G2A in panels A and B). As in the membrane binding analysis, 1–61 was even more efficiently incorporated into raft domains than wt and the behavior of 62–210 was comparable to that of G2A. The residual amounts of the 62–210 and G2A Nef variants in the DRM fraction in this particular experiment most likely reflects the slight contamination of the DRMs with non-raft material (see presence of TfR in DRM). Raft incorporation of Nef was primarily governed by the far N-terminus of Nef as 1–29 was readily recruited into raft clusters and DRMs (data not shown). Importantly, the KK Nef mutant was specifically excluded from DRMs (Fig. 4B) and, although still present in dotted structures at the plasma membrane, only minimal colocalization of KK could be detected with raft clusters in Jurkat T-cells (Fig. 4A). Mutation of the other N-terminal basic residues did not affect Nef’s association with raft clusters or DRMs (data not shown). Since all these Nef variants displayed comparable overall membrane association, the incorporation of Nef from HIV-1SF2 into raft domains is mediated specifically by the two N-terminal lysine residues. Together, these results reveal that membrane attachment and raft incorporation of Nef are governed by distinct signals and identify the two N-terminal lysines as specific determinant for the incorporation of Nef into detergent-resistant microdomains.

Downmodulation of select cell surface receptors requires differential avidity of membrane attachment of Nef

As a first functional correlate for the association of the various Nef mutants with cellular membranes, we tested their ability to downmodulate the surface exposure of CD4, MHC-I and CCR5 in TZM or CHO hCD4hCCR5 cells, respectively (Fig. 5)(Garcia and Miller, 1991; Michel et al., 2005; Schwartz et al., 1996). As described before (Michel et al., 2005), cell surface levels of the individual receptors were determined on living cells by flow cytometry and the mean fluorescence intensities were compared between untransfected (GFP-negative) cells and cells expressing medium to high levels of GFP or Nef.GFP. All values were plotted relative to the GFP negative control. As expected, wt Nef.GFP downmodulated all three receptors, albeit with different efficiency (80% for CD4, 38% for MHC-I and 63% for CCR5). The AxxA mutant, which is specifically defective in downmodulating MHC-I and CCR5, and the EDAA Nef variant, that is impaired in reducing cell surface CD4 levels, were used as specific controls. Of note, all Nef mutants with modifications in the N-terminal motifs retained full CD4 downregulation activity, while the activity of non-myristoylated G2A was partially reduced. Of note and in line with our results in HIV-1-infected T lymphocytes (Fackler et al., 2006), the G2A mutation did not have appreciable effects on Nef’s ability to downmodulate cell surface MHC-I. Consistently, modification of putative membrane interacting residues in the anchor domain also did not impair this Nef activity. A yet slightly different scenario was obtained when the activity of these Nef variants on cell surface CCR5 was analyzed: While myristoylation significantly contributed to this Nef effect (G2A reduced CCR5 levels by only 30%), tryptophane and arginine residues were fully dispensable. In contrast, simultaneous mutations of lysine and arginine residues in KR caused a slight but significant reduction of Nef’s ability to downregulate CCR5 (downregulation of CCR5 levels by 40%). Together, membrane association correlates to different degrees with the ability of Nef to downmodulate cell surface levels of CD4, MHC-I and CCR5.

Lysine residues in the N-terminus of Nef are critical for its association with Pak2 activity

To address the role of membrane association for signal transduction properties of Nef, we analyzed its ability to associate with cellular Pak2 kinase activity in Jurkat T lymphocytes.
Following immunosolation of various Nef.GFP fusion proteins and subsequent in vitro kinase (IVKA) reaction, Nef-associated phosphorylated Pak2 was visualized by autoradiography (Fig. 6, IVKA). Expression levels of the various Nef.GFP proteins were comparable as judged by Western blot analysis (Fig. 6, input). The bottom panel of Fig. 6 presents the quantification of the Nef-associated Pak2 activity based on the intensity of the autophosphorylated Pak2 band. It would be desirable to correlate the phospho-Pak2 levels with the amounts of immunoisolated Nef. However, this analysis was precluded for the Nef variants with lysine residues mutated because their co-migration with the antibody heavy chain prevented accurate quantification. In those cases where the normalization of phospho-Pak2 intensities for the amounts of Nef.GFP present in the immunoisolates was possible, relative differences similar to those presented were observed (data not shown). Expectedly, wt readily associated with robust amounts of Pak2 activity while the negative controls GFP and AxxA did not reveal specific Pak autophosphorylation products (24% and 20% activity). Of note, the association of G2A with Pak2 activity was also drastically impaired (16% activity). In contrast, tryptophane and arginine residues were entirely dispensable for the association of Nef with Pak2 activity and mutations of the arginines even increased the activity of Nef. This increase in association with Pak2 activity did not stem from an augmented raft incorporation of the R4 Nef variant (data not shown). Thus, the loss of these positively charged residues, possibly by affecting protein conformation, may somehow facilitate the interaction of Nef with Pak2 or other components of the Nef–Pak2 complex. In contrast, mutations of the lysine residues markedly reduced the association of Nef with active Pak2 (49% and 54% activity for KR and KK, respectively). Together, membrane attachment is a strict requirement for Nef’s ability to associate with Pak2 activity and the lysine residues in the N-terminus of Nef were identified as a novel determinant of this Nef activity.

**Virus replication and virion incorporation**

Our previous analyses demonstrated that the R4 mutant of SF2 Nef is readily incorporated into HIV-1 virions and displays wt activity in the enhancement of virion infectivity as well as in boosting HIV-1 replication in primary human T-lymphocytes (Fackler et al., 2006). To analyze the role of all N-terminal membrane targeting signals in these functional assays, isogenic viruses using the HIV-1NL4-3 proviral backbone were constructed that encode for the WR, KK and KR variants of SF2 Nef. A virus expressing wt SF2 Nef (wt) and a nef-deleted variant (Δnef) served as controls. Virus was produced in 293T cells following transfection of the indicated proviral DNAs and resulted in the production of comparable amounts of virus particles for all constructs (data not shown). Western blot analysis of transfected 293T cells revealed similar levels of expression for all Nef variants and comparable patterns of Gag processing (Fig. 7A). When HIV particles were pelleted from these cell culture supernatants (Fig. 7B), comparable amounts of virus particles were observed for all constructs (data not shown).
of wt as well as the WR, KK and KR Nef mutants were detected in virions. Thus, consistent with our previous findings on virion incorporation of the G2A variant of SF2 Nef (Fackler et al., 2006), neither the reduced membrane binding capacity of the KR mutant nor the reduced lipid raft incorporation of the Nef KK caused an appreciable reduction of Nef’s incorporation into virus particles. In contrast, marked differences were observed in the relative infectivity of virions in a single round of infection on TZM reporter cells (Fig. 7C). 36 h following infection with 0.5 ng p24CA, wt was approximately 10-fold more infectious than Δnef. Of note, the WR mutant increased virion infectivity as efficiently as wt, indicating that the tryptophanes and the arginines are dispensable for Nef’s effect on virion infectivity. In contrast, both Nef variants in which K4 and K7 were mutated (KK and KR) were significantly impaired in enhancing HIV-1 virion infectivity. This effect was most pronounced for KK Nef that was almost completely inactive in this assay. These findings suggest that incorporation of Nef into lipid rafts may be instrumental for its effect on virion infectivity. Finally, the effect of these Nef variants on HIV-1 replication in primary human PBMCs was analyzed. According to previous studies (Fackler et al., 2006; Welker et al., 1998), resting PBMCs were infected, subsequently activated by PHA/IL-2 for 3 days and HIV-1 replication was monitored over time (Fig. 7D). Under these experimental conditions, HIV-1 replication was significantly reduced in the absence of Nef during the first two days and subsequently reached levels of p24 production comparable to those of wt (Fig. 7D, compare wt and Δnef). Interestingly, all three Nef variants analyzed failed to increase HIV-1 replication over the level of Δnef during this time period and were thus considered inactive in augmenting virus replication in primary human T lymphocytes.

**Discussion**

The present study identifies membrane binding determinants of HIV-1gp2 Nef in human cells and unravels their individual contribution to Nef’s biological activity. Based on the microscopic and biochemical analyses presented, membrane association of the viral protein requires its N-terminal myristoylation, basic arginine and lysine residues near the N-terminus and at least one additional motif in the N-terminal anchor domain. Importantly, two N-terminal lysine residues were found to be critical also for the incorporation of Nef into lipid rafts. Host cell signal transduction as well as most receptor sorting processes were modulated with maximum efficiency only by Nef variants with full membrane association capacity. As summarized in Table 1, individual Nef functions were
sensitive to various extend to the reduction of its membrane association or raft incorporation. While we cannot exclude that the mutations introduced cause effects in addition to the observed alterations in membrane association, these findings imply membrane interactions and segregation into raft-like domains as a critical regulatory mechanism for Nef’s activities in the infected host.

The data presented reveal that the N-terminal anchor is sufficient for the association of Nef with cellular membranes. Indeed, the Nef anchor fragments of various length tethered their GFP fusion moiety to membranes with even higher efficiency than the full-length Nef protein. Thus, the core domain of Nef, presumably because of its relatively large mass, is not required for membrane association or raft incorporation. While we cannot exclude that the mutations introduced cause effects in addition to the observed alterations in membrane association, these findings imply membrane interactions and segregation into raft-like domains as a critical regulatory mechanism for Nef’s activities in the infected host.

Myristoylation and N-terminal lysine and arginine residues were critical for Nef’s membrane association and subcellular localization. In contrast, despite the potential to mediate protein–membrane interactions (Wimley and White, 1996), tryptophane and methionine residues near the N-terminus of the viral protein were dispensable for membrane association and functionality of the viral protein in human cells. Based on our analysis with N-terminal Nef fragments and Nef deletion mutants, Nef contains at least one additional signal in between amino acids 40–61 that contributes to its plasma membrane localization. This stretch bears multiple charged amino acids that form a short alpha helix (Geyer et al., 1999). Some of these charged residues are reportedly required for the ability of several HIV-1 Nef proteins to modulate cell surface CD4 but not MHC-I and MHC-II molecules (Aiken et al., 1996; Casartelli et al., 2003; Iafrate et al., 1997; Mangasarian et al., 1999; Schindler et al., 2003). However, these studies did not

### Table 1

Summary of localization and activities of the HIV-1SF2 Nef proteins analyzed

<table>
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<tr>
<th>Nef variant</th>
<th>Fractionation</th>
<th>DRM/raft association</th>
<th>CD4 ↓</th>
<th>MHC-I ↓</th>
<th>CCR5 ↓</th>
<th>Pak2 association</th>
<th>Virion incorporation</th>
<th>Infectivity enhancement</th>
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<tr>
<td>G2A</td>
<td>86±7</td>
<td>14±7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Δ12–39</td>
<td>76±6</td>
<td>24±6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+/− k</td>
<td>− b</td>
<td>− b</td>
<td>− b</td>
</tr>
<tr>
<td>WR</td>
<td>75±8</td>
<td>25±8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>KK</td>
<td>75±12</td>
<td>25±12</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NR</td>
<td>96±2</td>
<td>4±2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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+ indicates activity comparable to the wt protein, – designates loss of function, +/− intermediate activity and ++ increased activity relative to wt HIV-1SF2 Nef. Nef fragments were not tested in functional assays because of the lack of relevant domains/motifs that mediate the activity of the full-length Nef protein.

- Baur et al. (1997).
- Fackler et al. (2006).

The data presented reveal that the N-terminal anchor is sufficient for the association of Nef with cellular membranes. Indeed, the Nef anchor fragments of various length tethered their GFP fusion moiety to membranes with even higher efficiency than the full-length Nef protein. Thus, the core domain of Nef, presumably because of its relatively large mass and volume, the lack of specific membrane targeting signals and the presence of specific sorting motifs, somewhat counteracts the membrane association of the N-terminus of Nef, thereby resulting in the steady state distribution of Nef observed. Alternatively, Nef may undergo a conformational change between its membrane-bound and cytosolic form such that the N-terminal fatty acid interacts with the core domain to facilitate its solubilization as recently suggested (Breuer et al., 2006). Truncation of the core domain would hence lead to increased exposure of the myristate. In line with this scenario, the myristoylated anchor of Nef is significantly less soluble in vitro than the myristoylated full-length protein (Breuer et al., 2006; Geyer et al., 1999). Of note, despite the use of different biochemical procedures by several investigators, the overall proportion of membrane-associated Nef protein is consistently estimated to approx. 30% in different mammalian cells using Nef overexpression or HIV infection (Bentham et al., 2006; Fackler et al., 1997, 2006; Kaminkich et al., 1994; Niederman et al., 1993). In contrast, the microscopic analysis of Nef’s subcellular localization in fibroblasts gave variable results ranging from a relatively diffuse cytoplasmic distribution to a marked punctuate staining and the association with intracellular membranous structures (Bentham et al., 2006; Craig et al., 2000; Fackler et al., 2006; Greenberg et al., 1997; Keppeler et al., 2005) but without pronounced localization of Nef at the plasma membrane. The use of live cell imaging of Nef.GFP in the present study circumvented artifacts that can be induced by cell fixation. This analysis revealed a distribution that is consistent with the biochemical analysis and strongly supports the notion that substantial amounts of Nef are membrane-associated including a pronounced localization at the plasma membrane in intact human cells. The identity of the intracellular Nef-positive membrane compartment(s) remains unclear. We and others (Blagoveshchenskaya et al., 2002; Michel et al., 2006; Pigu et al., 2000) have shown a significant overlap of Nef with the trans-Golgi network (TGN). However, partial overlap of Nef with endosomal membrane compartments can also be conserved at the densely packed perinuclear area in fibroblasts (data not shown). The limited resolution of confocal microscopy therefore warrants a more refined analysis by, e.g., electron microscopy to unambiguously define these membranous compartment(s).
investigate whether the mutations introduced affect Nef’s membrane association or subcellular localization and the characterization of corresponding mutants has not been reported for SF2 Nef. Unfortunately, similar to a previous report (Mangasarian et al., 1999), our attempts to analyze the relevance of these amino acids for membrane association and function of SF2 Nef were hampered by the instability of all proteins tested with deletions or point mutations of the candidate charged residues. Thus, in addition to its role in subcellular targeting of Nef, this alpha helix likely plays an important role for the overall structural organization and thus stability of Nef in human cells.

The functional characterization of the basic cluster in SF2 Nef confirms and extends a recent study by Bentham and colleagues (2006) on Nef from NL4-3. Both studies agree that the lysine residues alone do not play a significant role in Nef’s membrane attachment per se and demonstrate that the lysine and arginine residues together contribute more to Nef’s association with cell membranes than the myristoyl anchor. However, while the arginines were found to have a significant impact on membrane binding in biochemical analyses for NL4-3 Nef, the R4 SF2 Nef mutant behaved almost like wt in our hands. This difference likely reflects the variability between these Nef alleles that may be determined by the insertion of four amino acids in the anchor domain of SF2 Nef (Geyer et al., 2001). Similarly, when judged by confocal microscopy, the R4 mutation significantly increased a diffuse cytosolic localization of SF2 Nef in the present study while Bentham et al. (2006) observed a translocation of the R4 mutant of NL4-3 Nef from the plasma membrane and the ER/Golgi compartment to mitochondria. Such allelic differences are also apparent when the role of the lysine and arginine residues was analyzed for the association of Nef with GagPol proteins (Krautkramer et al., 2004; Sol-Foulon et al., 2004) that were present with up to 50% in detergent-resistant micro-domains. This approach demonstrated that augmented raft incorporation of Nef emphasizes its ability to associate with Pak2 kinase activity but does not affect its ability to downmodulate cell surface CD4 and MHC-I receptors or to boost HIV infectivity and replication. Thus, while the Nef–Pak2 association was found to benefit from Nef’s raft incorporation, this localization was not limiting for these other Nef activities. The functional analysis of the KK variant extends these earlier findings by demonstrating that raft incorporation is necessary for the association of Nef with active Pak2 and fully dispensable for its effects on cell surface CD4, MHC-I and CCR5. Of note, raft exclusion of Nef interfered with its ability to augment virion infectivity, which is in line with an involvement of raft domains in this Nef activity (Zheng et al., 2001). However, the incorporation of a small subpopulation of Nef in raft domains appears sufficient to imprint the increase in infectivity as further raft enrichment of Nef does not markedly accentuate its ability to enhance particle infectivity (Sol-Foulon et al., 2004). Consistent with our previous findings (Fackler et al., 2006), infectivity enhancement does not correlate with the incorporation of Nef into infectious virions, which also occurs in a raft-independent fashion. This may be surprising given that HIV particles preferentially bud from lipid raft like domains and the resulting virions are highly enriched in raft lipids (Brugger et al., 2006). Since neither myristoylation (Fackler et al., 2006) nor raft targeting is a prerequisite for virion incorporation, membrane association is highly unlikely to account for the presence of SF2 Nef in HIV-1 particles. Rather, protein interactions such as the association of Nef with GagPol (Costa et al., 2004) might mediate its virion incorporation. However, as the presence of Nef in HIV-1 particles is neither necessary nor sufficient to mediate Nef’s effects on particle infectivity and replication, the biological relevance of virion-associated Nef remains uncertain. Together, these findings
support a model in which raft incorporation regulates the biological activity of Nef where raft-associated Nef is devoted to at least some of the signaling activities and to infectivity enhancement while the modulation of intracellular receptor transport is governed by the non-raft resident Nef population.

Importantly, membrane association did not correlate equally well with all Nef activities analyzed. As discussed above, the association of Nef with active Pak2 is very sensitive to alterations of Nef’s membrane association, presumably reflecting the requirement of lipid raft incorporation for the assembly of the Nef–Pak2 complex (Krautkramer et al., 2004; Pulkkinen et al., 2004; Raney et al., 2005). In contrast, effects of Nef on cell surface receptor modulation required less pronounced membrane association. Interestingly, CD4 downregulation was efficiently mediated by the KR mutant that only retains minimal membrane affinity while the lack of myristoylation, that affects Nef’s overall membrane association to a lesser extent, was significantly impaired in this activity. This indicates that myristoylation, e.g., by affecting the overall structural conformation of Nef, may be important for processes other than membrane binding that are relevant for its biological activity. As Nef is thought to downmodulate CD4 by various mechanisms that involve the interaction of the viral protein with the CD4 cytoplasmic tail (Aiken et al., 1994; Lama, 2003; Rose et al., 2005), downmodulation could, in principle, be envisioned without intrinsic membrane binding of Nef (Bentham et al., 2003). The latter might also apply to downmodulation of MHC-I by SF2 Nef which, consistent with our recent results in HIV-1-infected T lymphocytes (Fackler et al., 2006), did not require myristoylation or presence of the basic cluster and was thus the least dependent on membrane association of all Nef activities analyzed herein. This might reflect that the effects of Nef on MHC-I surface presentation involve the formation of a complex between Nef, MHC-I and AP-1 (Roeth et al., 2004; Williams et al., 2005), which might not require intrinsic membrane binding by Nef. Of note, relative to the effects on MHC-I, the downmodulation of CCR5 was more sensitive to a reduction of Nef’s membrane association. We previously reported that downmodulation of both, MHC-I and CCR5 require identical signature motifs in Nef (Michel et al., 2005). The differential requirement for membrane attachment provides a first indication that the mechanisms utilized by Nef to downmodulate these surface receptors may not be fully identical and suggests that relative requirements for membrane association may be employed to dissect the cellular pathways targeted by individual Nef activities. Finally and consistent with previous findings for NL4-3 Nef (Welker et al., 1998), the positive effects of SF2 Nef on HIV-1 replication in primary human T lymphocytes were very sensitive to modifications in its membrane targeting and raft incorporation determinants. This implies that multiple, both raft-dependent and -independent, activities are exerted by Nef simultaneously to achieve the optimization of virus replication thought to be crucial for its role as pathogenicity factor in vivo.

Together, the findings presented support the idea that Nef undertakes a journey through the infected cell where multiple targeting signals determine its localization and thus its biological activity. Co-translational myristoylation of Nef allows prompt membrane targeting by the anchor domain that contains the myristoyl moiety, basic residues and at least one additional targeting determinant. Presumably following initial transport to the plasma membrane, Nef segregates into raft-resident and non-resident subpopulations with distinct biological properties. Subsequently, sorting motifs in the core domain of Nef cause the tethering to a variety of intracellular vesicular organelles that might mediate at least some of Nef’s effects on intracellular transport of surface receptors. The mechanisms involved in the specificity of Nef to select cellular membranes and its initial transport to the plasma membrane as well as the potential biological role of non-membrane-associated Nef will be subject to future studies.

Materials and methods

Cells and reagents

HeLa, 293T, TZM (JC53BL) and TZM clone 13 cells (subcloned for high expression of MHC-I by fluorescence-activated cell sorting) were maintained in DMEM high medium (from GIBCO, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany), 1-glutamine and antibiotics (both from GIBCO). Jurkat TAg (expressing the large T antigen of SV-40 and referred to as Jurkat T-cells) and CHO hCD4hCCR5 (stably expressing hCD4 and hCCR5, described in Kepler et al., 2005) cells were cultured in RPMI 1640 (from GIBCO) medium supplemented accordingly. RPMI for PBMC cultures was supplemented with 10% FCS, 0.1% Glutamax (Invitrogen), 0.1% 1 M HEPES (pH 7.4) and antibiotics.

Expression plasmids

Nef from HIV-1sf2 was used throughout the study. Constructs encoding wild-type HIV-1sf2 Nef and the G2A, Δ12–39, EDAA and AxxA mutants cloned into pEGFP-N1 (Invitrogen, Karlsruhe, Germany) have been described (Keppeler et al., 2005; Krautkramer et al., 2004). The deletion mutants were generated by PCR with primers introducing BglII and BshTI sites 5′- and 3′ of the nef amplicon, respectively, and were subcloned in the BglII/BshTI digested pEGFP-N1 vector. The expression plasmids for the WW, WM, WR, R4, KK and KR Nef variants as Nef.GFP fusion proteins were generated by site-directed mutagenesis and subcloning. Isogenic proviral constructs expressing various nef genes were generated by PCR with primers introducing BglII and BshTI sites 5′- and 3′ of the nef amplicon, respectively, and were subcloned in the BglII/BshTI digested pEGFP-N1 vector. The expression plasmids for the WW, WM, WR, R4, KK and KR Nef variants as Nef.GFP fusion proteins were generated by site-directed mutagenesis and subcloning. Isogenic proviral constructs expressing various nef genes were constructed in the backbone of the HIV-1NL4-3 proviral clone as described earlier (Fackler et al., 2006). The complete nucleotide sequence of all nef inserts of these proviral clones and the Nef.GFP expression plasmids was verified by sequencing of both DNA strands.

Western blotting

For Western blot analysis, samples were boiled in SDS Sample Buffer, separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane. Protein detection was performed
following incubation with appropriate first and secondary antibodies using the super signal pico detection kit (Pierce, Bonn, Germany) according to the manufacturer’s instructions. GFP constructs were detected with anti-GFP mouse monoclonal antibody (BD Living Colors). CA and Nef were detected using the polyclonal rabbit serum CA1 (Müller et al., 2004) and the polyclonal sheep serum Arp444 (Coates et al., 1997), respectively.

**Live cell imaging**

To visualize the subcellular localization of Nef, HeLa cells were seeded on eight-chambered coverglasses (Lab-Tek, Nunc, Rochester, New York) and transfected with Lipofectamine2000 (Invitrogen) the next day. 24 h post-transfection, cells were analyzed by confocal microscopy using an LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Confocal images were processed with AdobePhotoshopCS2 software (Adobe Systems, Mountain View, CA).

**Subcellular fractionation**

Subcellular fractionation was performed using slight modifications of a previously described protocol (Alland et al., 1994). In brief, 293T cells were transfected with Metafectene. 24 h post-transfection, cells were resuspended in hypotonic buffer (10 mM Tris, pH 7.4, 0.2 mM MgCl2, protease inhibitor cocktail) and homogenized by pipetting up and down for 50 times. Postnuclear supernatants were adjusted to 0.25 M sucrose and 1 mM EDTA and centrifuged at 100,000×g for 50 min. Volumes of pellets (P) and supernatants (S) were equalized using RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1% Triton-X 100, 0.1% SDS). 6× SDS Sample Buffer was added and aliquots corresponding to 3% of the P and S fractions or of the total cell lysate (IN) were analyzed by Western Blotting using antibodies against Nef, TIR (mAb H68.4, Zymed Laboratories Inc., South San Francisco, CA) and 14-3-3 (BioRad). For quantification, films were scanned and the relative band intensities were determined with the QuantityOne software (BioRad) after background subtraction. The combined signal intensity from the P and S fractions of each sample was set to 100% to yield the relative distribution of proteins between the P and S fractions.

**Raft flotation**

293T cells were transfected with Lipofectamine 2000 (Invitrogen) and assay was performed using slight modifications of a previously described protocol (Krautkramer et al., 2004). In brief, 24 h post-transfection, 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 pipetting up and down for 50 times, lysates were adjusted to 900 μl of 40% Optiprep (Life Technologies, Karlsruhe, Germany) and overlaid with 2.5 ml of 28% Optiprep and 0.5 ml of TXNE. After ultracentrifugation (SW60 Ti rotor, 35.000 rpm, 4 °C, 3 h) eight fractions of 500 μl were collected from the top. 2.5% of the detergent-resistant membrane fraction (fraction 2) and the pooled non-raft fractions (fractions 7 and 8), respectively, were analyzed together with 0.5% of the unfractionated cell lysate by Western Blotting.

**Flow cytometry**

CHO hCD4hCCR5 or TZM clone13 cells were transfected with Nef:GFP expression constructs, respectively. Cells were harvested 24 h post-transfection and CHO hCD4hCCR5 cells were stained with anti-hCD4-APC and anti-hCCR5-APC (BD) antibodies and TZM clone 13 cells with anti-MHC-I (W6/32-FITC, Sigma-Aldrich, St. Louis, MO), respectively. All cell surface molecules were stained at 4 °C for 30 min and samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA).

**In vitro kinase assay (IVKA)**

In vitro kinase assay was performed as described earlier (Krautkramer et al., 2004). Briefly, 1 × 10² Jurkat T-cells were electroporated and 24 h later lysed in KEB (137 mM NaCl, 50 mM Tris–HCl pH 8, 2 mM EDTA, 0.5% Nonidet P-40, Na₃VO₄, protease inhibitors). Postnuclear supernatants were immunoprecipitated with anti-GFP antibodies. After 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 MgCl₂) containing 10 μCi of [γ-32P]ATP (Amersham, Freiburg, Germany) per reaction. After incubation for 5 min, samples were washed and bound proteins were separated by SDS–PAGE and subjected to autoradiography. Quantification was performed on a phosphoimager (BioRad, Munich, Germany) by using QuantityOne software (BioRad).

**Raft clustering**

24 h post-transfection, Jurkat T-cells were incubated with 25 μg of Alexa 555- or Alexa 594-conjugated CTx/ml, respectively, in 0.1% BSA/PBS for 30 min on ice. After washing, 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 glass coverslips, fixed with 3% paraformaldehyde-PBS, washed and mounted with Histoprime (Histogel, Linaris, Germany). Fluorescence microscopy images were acquired with an LSM 510 confocal laser scanning microscope (Zeiss microscope with a 100× oil-immersion objective equipped with a CCD camera) and processed with AdobePhotoshopCS2 software (Adobe Systems, Mountain View, CA).

**Virus stocks and virion infectivity**

Virus stocks were generated by transfection of proviral HIV plasmids into 293T cells using Metafectene (Biontex Systems, Mountain View, CA).
Laboratories, Munich, Germany). Two days after transfection, culture supernatants were harvested. The CA concentration of these virus stocks was determined by CA antigen enzyme-linked immunosorbent assay (ELISA) as described (Muller et al., 2004). The relative infectivity of HIV-1 particles was determined by CA ELISA and a standardized 96-well TZM blue cell assay as described (Keppeler et al., 2005). Briefly, infections were carried out in triplicates with 0.5 ng CA input virus. 36 h post-infection, cells were fixed, stained for β-galactosidase activity and the number of blue cells was determined by microscopy.

**Virion incorporation**

For the analysis of virion incorporation of Nef, 400 µl of cell culture supernatants containing infectious virions were placed on top of 80 µl of a 20% sucrose solution. After ultracentrifugation (TLA45 rotor, 44,000 rpm, 4 °C, 60 min), the pellets were resuspended in 6× Sample Buffer. Volumes corresponding to 100 or 5 ng CA were then analyzed by Western blotting with antibodies against Nef and CA, respectively.

**Replication in primary human peripheral blood lymphocytes (PBL)**

To analyze the effects of Nef on HIV replication in primary human T lymphocytes, peripheral blood mononuclear cells were isolated from healthy donors by Ficoll gradients using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). For infection, cells were thawed and kept in bulk cultures in RPMI, 10% FCS at 1 × 10⁶ cells/ml over night. 1 × 10⁵ cells/well were isolated from healthy donors by Ficoll gradients using ultracentrifugation (TLA45 rotor, 44,000 rpm, 4 °C, 60 min), the pellets were resuspended in 6× Sample Buffer. Volumes corresponding to 100 or 5 ng CA were then analyzed by Western blotting with antibodies against Nef and CA, respectively.

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