Intrinsic properties and plasma membrane trafficking route of Src family kinase SH4 domains sensitive to retargeting by HIV-1 Nef

Amanda J. Chase, Rebecka Wombacher, and Oliver T. Fackler

From the Department of Infectious Diseases, Center for Integrative Infectious Disease Research (CIID), Integrative Virology, University Hospital Heidelberg, Im Neuenheimer Feld 344, 69120 Heidelberg, Germany

Received for publication, March 9, 2018 Published, Papers in Press, March 27, 2018, DOI 10.1074/jbc.RA118.002794

The HIV type 1 pathogenicity factor Nef enhances viral replication by modulating multiple host cell pathways, including tuning the activation state of infected CD4 T lymphocytes to optimize virus spread. For this, Nef inhibits anterograde transport of the Src family kinase (SFK) Lck toward the plasma membrane (PM). This leads to retargeting of the kinase to the trans-Golgi network, whereas the intracellular transport of a related SFK, Fyn, is unaffected by Nef. The 18-amino acid Src homology 4 (SH4) domain membrane anchor of Lck is necessary and sufficient for Nef-mediated retargeting, but other details of this process are not known. The goal of this study was therefore to identify characteristics of SH4 domains responsive to Nef and the transport machinery used. Screening a panel of SFK SH4 domains revealed two groups that were sensitive or insensitive for trans-Golgi network retargeting by Nef as well as the importance of the amino acid at position 8 for determining Nef sensitivity. Anterograde transport of Nef-sensitive domains was characterized by slower delivery to the PM and initial targeting to Golgi membranes, where transport was arrested in the presence of Nef. For Nef-sensitive SH4 domains, ectopic expression of the lipoprotein binding chaperone Unc119a or the GTPase Arl3 or reduction of their endogenous expression phenocopied the effect of Nef. Together, these results suggest that, analogous to K-Ras, Nef-sensitive SH4 domains are transported to the PM by a cycle of solubilization and membrane insertion and that intrinsic properties define SH4 domains as cargo of this Nef-sensitive lipoprotein binding chaperone–GTPase transport cycle.

Nef is a 25- to 34-kDa myristoylated accessory protein encoded by HIV-1, HIV-2, and SIV. Although Nef is not needed for virus replication in cell culture, it potently increases viral replication in a physiological environment, thus serving as a pathogenesis factor that accelerates disease progression (1–3). Nef acts to mediate functions via various interactions with host cell proteins, thereby inducing a variety of changes in central intracellular transport and signaling pathways of HIV-infected cells (4, 5). This includes reducing cell surface densities of transmembrane receptors by molecular mechanisms that affect endocytosis, anterograde transport, and/or protein stability (6–10). By modulating surface exposure of cell surface receptors, such as major histocompatibility complex I and II, CD4, chemokine receptors, co-stimulatory molecules such as CD80 and CD86, tetraspanins, and natural killer cell ligands, Nef acts to evade host cell immune responses and prevent superinfection of infected cells (7–15). In addition, Nef affects T cell activation and the responsiveness of T lymphocytes to TCR signaling by modifying vesicular transport and actin dynamics (16–24). In such a way, activation-induced cell death is reduced, thus prolonging the survival of productively infected cells (25).

In addition to transmembrane receptors, Nef alters the subcellular localization of peripheral membrane proteins such as the Src family kinase (SFK) Lck, which is retargeted by Nef from the plasma membrane (PM) to the trans-Golgi network (TGN) in HIV-infected CD4 T cells (19, 26–28). As Lck is the master switch for TCR signaling (20), this is one means by which Nef disrupts early TCR signaling and influences a select array of intracellular signaling events to optimize HIV replication. Along with Lck, a closely related SFK, Fyn, also acts to regulate early TCR signal transduction (29), but its subcellular localization is unaffected by Nef (27), illustrating that Nef uses a highly specific mechanism to relocalize Lck from the PM to the TGN. SFKs are composed of Src homology (SH) domains 1–4, where the SH4 domain acts as membrane anchor, the SH2 and SH3 domains are protein interaction modules, and the SH1 domain bears kinase activity (30). By virtue of its acylation (myristoylation and/or palmitoylation) and, in the case of Src, in concert with positively charged amino acids, the 18-amino acid N-terminal SH4 domain provides membrane anchoring and determines the subcellular localization and trafficking route of the SFK (31–36). Although the subcellular localization of SFKs such as Lck or Fyn is regulated by a complex system of anterograde transport to and internalization from the PM, little is known about the machinery and regulation of the transport.
pathways involved. Clearly distinct from the classical secretory pathway employed by transmembrane proteins for PM delivery, different SFKs with distinct lipid modifications of the SH4 domain appear to utilize varied PM delivery pathways (33, 37). Lyn, for example, traffics directly to the PM following synthesis (37, 38). Src, on the other hand, is rapidly exchanged between the PM and late endosomes or lysosomes (39). Other SFKs, such as Lyn and Yes, initially enter the Golgi system after biosynthesis, where they are palmitoylated and traffic to the PM via vesicle-mediated transport (40). On the molecular level, several factors important for Lck anterograde transport have been identified, including myelin and lymphocyte protein (MAL), formin actin nucleator INF2, uncoordinated 119 (Unc119), and Rab11, which are thought to facilitate anterograde transport of Lck-containing vesicles toward the PM (23, 41–46).

Similarly, little information is available about how Nef selectively impairs anterograde transport of Lck but not Fyn. We previously defined that the SH4 domain of Lck is sufficient for Nef-mediated retargeting from the PM to the TGN and that the SH3 domain binding motif in Nef is required for this effect (18, 27, 47). Nef does not physically interact with the Lck SH4 domain, and a mutation in Nef that prevents its localization to the TGN site of Lck accumulation in the presence of the viral protein retains the ability to retarget Lck (18, 27, 47). Thus, Nef appears to induce Lck relocalization by an indirect mechanism that requires Nef itself to undergo dynamic vesicular transport cycles (49). This mechanism applies to newly synthesized protein during its anterograde transport to the PM and is paralleled by a marked reduction in association of Lck with detergent-resistant membrane (DRM) microdomains but not with altered overall membrane association (47). Together, these results suggest that Nef interferes with anterograde transport of specific membrane microdomain platforms by blocking their transport and/or recruitment of cargo molecules.

Overexpression of Unc119a releases Lck from the TGN after retargeting by Nef (27). Surprisingly, expression of Unc119a, which is known to interact with SFKs via the SH2 and SH3 domains to activate the kinase (42, 50), did not restore the normal distribution of Lck to the PM but instead resulted in diffuse cytoplasmic staining, indicative of a soluble protein (27). This was a puzzling observation, as Lck maintained the palmitoylation of its SH4 domain under these conditions, a modification that requires shielding from the aqueous cytoplasm. This may now be explained by the recent characterization of Unc119a as a member of a protein family that acts as lipoprotein binding chaperones (LPCs) to solubilize acylated peripheral membrane proteins by binding their fatty acid in a hydrophobic pocket (51–53). LPC activity has been reported for Unc119a, Unc119b, heme oxygenase 2 (HO2), and phosphodiesterase 68 (PDE6δ) (51, 54–58). Among these, PDE6δ is established to facilitate, by virtue of its LPC function, an unconventional anterograde transport pathway for K-Ras that involves consecutive steps of membrane insertion and solubilization (54, 59, 60). K-Ras is predominantly localized to the PM at steady state, although thermodynamics favors localization at endomembranes, in part because of the transient nature of palmitoylation (61, 62). When the prenylated moiety of K-Ras is protected from the cytosol upon binding to the hydrophobic pocket of PDE6δ, soluble K-Ras–PDE6δ complexes are subject to cytoplasmic diffusion (54). This soluble state can be reversed by reinsertion of K-Ras into membranes upon formation of a ternary complex with Arf-like (Arl) GTPase. Arl2 GTP hydrolysis provides the energy for a conformational change that releases K-Ras from the LPC and results in its enrichment at perinuclear membranes, with a preference for recycling endosomes. K-Ras associated with recycling endosomes is transported to the PM, from where it is again trafficked to the TGN via the endomembrane system (59, 60). As an LPC, Unc119a is able to bind the fatty acid of myristoylated and palmitoylated peripheral membrane proteins (51, 55, 58, 63). Furthermore, Arl2 and the closely related Arl3 GTPases can interact with Unc119a, causing the hydrophobic binding pocket to widen and cargo to be released (55). The binding affinity of the cargo to the Unc119a hydrophobic pocket determines whether Arl2 or Arl3 acts as the GTPase for release (64). Whether Unc119a employs this mechanism to promote dynamic transport cycles of SFKs remains to be established.

The goal of this study was to define the parameters that govern the specificity of HIV-1 Nef for TGN retargeting of SFK SH4 domains and to investigate whether LPC transport machinery is involved in this process. Our results indicate that the sensitivity of SH4 domains to TGN retargeting by Nef can be determined by a single amino acid and is characterized by usage of an LPC-based PM delivery pathway involving the LPC Unc119 and the GTPase Arl3.

Results

**Nef targets a subset of SFK SH4 domains to the TGN**

We previously found that the N-terminal SH4 domain, consisting of 18 amino acids (N18), is necessary and sufficient for Nef-mediated retargeting of Lck but not Fyn to the TGN (47). To gain more insight into the mechanism of Nef action in this process, we first tested whether the molecular surfaces or functional motifs by which Nef induces TGN retargeting are the same for the Lck SH4 domain (LckN18) compared with the full-length counterpart. We transiently expressed a panel of Nef.RFP WT or mutant proteins, along with LckN18 fused to GFP (LckN18.GFP), in Jurkat T (JTA) cells (Fig. 1, A and B, and Fig. S1A). As established previously (27, 47), we analyzed the steady-state subcellular localization of LckN18.GFP by confocal microscopy (Fig. 1A), and the number of cells with pronounced TGN localization of LckN18.GFP (frequency of accumulation) was determined (Fig. 1B). As expected, LckN18.GFP was predominantly localized at the PM when expressed with RFP control (Fig. 1B, 10.4% ± 0.6% of cells with pronounced intracellular enrichment of LckN18.GFP) and was found to be retargeted to an intracellular compartment when WT Nef.RFP was expressed (80.4% ± 4.3% of cells). The Nef mutant G2A, in which the myristoylation acceptor glycine at position 2 is replaced by an alanine to reduce its membrane association (4, 65, 66), failed to retarget LckN18.GFP (23.2% ± 2.4%). Similarly, the Nef mutant AXXA, in which the two central prolines of the SH3 interaction motif are replaced by alanine to disrupt interactions with SH3 domain-containing proteins (67), failed...
Block of SH4 domain anterograde transport by HIV-1 Nef

Figure 1. A specific subset of SH4 domains is retargeted to the TGN by Nef. A, LckN18.GFP and RFP, Nef.RFP, or the indicated Nef mutants were transiently expressed in JTAg cells. Subcellular localization was analyzed, and representative confocal images are shown. Arrowheads indicate TGN localization of LckN18.GFP. Scale bar = 2 μm. B, quantification of the frequency of cells with Nef-mediated TGN retargeting. 100 cells were analyzed from three independent experiments, and the mean percentage of cells that were retargeted to the TGN by the RFP control (white column) or Nef.RFP or the various mutants (black columns) are shown ± S.D. C, JTAg cells transiently expressing the indicated N18.GFP constructs and Myc or Nef.myc were stained for the TGN marker TGN46. Representative images are shown for all eight SH4 domains. Arrowheads indicate positive for Nef.myc. Scale bar = 2 μm. D, mean percentage of SH4 domain retargeting to the TGN for three independent experiments ± S.D. in cells co-expressing Myc (black columns) or Nef.myc (white columns).

Several Nef mutants retained the ability to retarget LckN18.GFP to the TGN, excluding these respective motifs as essential determinants of retargeting. These included KKAA (71.3% ± 5.4%), a lysine-to-alanine exchange at position 4 and 7 that disrupts Nef incorporation in DRM microdomains (65); EDAA (79.2% ± 5.1%), a mutation in the diacidic interaction motif in the C-terminal flexible loop of Nef that results in a defect in CD4 down-regulation and enhancement of virion infectivity (68); F195A (75.4% ± 1.8%), a mutation that disrupts Nef association with Pak2 that is important for inhibition of T cell actin dynamics and motility (69–72); and Δ12–39 (78.0% ± 4.1%), a deletion of the interaction site for the Nef-associated kinase complex that interferes with HIV transcription and release of extracellular vesicles (73–76) and has recently been shown to be involved in antagonism of particle infectivity restriction by SERINC5 (77). These results match the mapping of molecular Nef determinants for TGN retargeting of full-length Lck (18), suggesting that Nef affects the subcellular localization of N18 and full-length Lck by the same molecular surfaces.

In contrast to Lck, subcellular localization and transport of the closely related SFK Fyn is not altered by Nef, and the reasons for this specificity are unclear (47). Along with Fyn and Lck, there are seven other closely related members of the SFKs. To gain insight into the molecular signature in SH4 domains determining sensitivity to Nef, we screened these SH4 domains for TGN retargeting by Nef. To that end, Nef.Myc or the Myc vector control was transiently expressed in JTAg cells, along with the different SH4 domains of SFKs or the Leishmania protein Haspb (N18.GFP). Localization of the SH4 domains was observed at steady state (Fig. 1C and Fig. S1B), and the frequency of retargeting to the TGN by Nef.Myc or the Myc control was compared for each SH4 domain (Fig. 1D). To determine whether relocalization of SH4 domains led to retargeting at the TGN for other SH4 domains, cells were stained for the TGN marker TGN46. As seen previously, LckN18.GFP was retargeted to the TGN by Nef (80.4% ± 5% of cells with prominent TGN localization) whereas FynN18.GFP was not (8.5% ± 5.6%). Interestingly, the SH4 domains LynN18.GFP (78.1% ± 2.0%), SrcN18.GFP (72.1% ± 2.1%), HaspbN18.GFP (76.0% ± 0.6%), and HckN18.GFP (75.1% ± 0.4%) were retargeted to the TGN by Nef with similar efficiency as LckN18.GFP. In contrast, the distribution of YesN18.GFP (12.1% ± 4.2%) and FgrN18.GFP (14.6% ± 4.5%) was as unaffected by Nef as FynN18.GFP.
Importantly, this pattern of Nef-mediated retargeting of a specific subset of SH4 domains was also seen in primary T cells (Fig. 2, A and B, and Fig. S2A). LckN18.GFP, LynN18.GFP, FynN18.GFP, and YesN18.GFP were primarily found at the PM of primary T cells when co-expressed with RFP (8.7% ± 1.7%, 10.0% ± 1.8%, 9.4% ± 2.4%, and 6.4% ± 3.7%, respectively) or the Nef mutant AXXA (6.1% ± 1.9% for LckN18.GFP). Co-expression of WT Nef.RFP induced prominent retargeting of LckN18.GFP (67% ± 4.5%) and LynN18.GFP (68.6% ± 2.4%), whereas FynN18.GFP (16.7% ± 3.0%) and YesN18.GFP (8.0% ± 3.9%) were not retargeted by Nef. Furthermore, the same specificity of retargeting of Lck and Lyn SH4 domains, but not Fyn or Yes SH4 domains, was observed for additional Nef variants from HIV-1 (NL4.3), HIV-2 (Cbl23), and SIV (mac239) (Fig. 2, C and D, and Fig. S2B). Thus, lentiviral Nef proteins re-target only a specific subset of SFK SH4 domains to the TGN in primary CD4+ T cells and T cell lines, whereas other SH4 domains are resistant to Nef.

**Overexpression and silencing of LPC transport machinery phenocopies the effects of Nef on SFK SH4 domain localization**

Because the LPC Unc119a is involved in PM delivery of Lck (41, 42), we wanted to know whether Unc119a also has an effect on the transport of other SH4 domains. To that end, Unc119a.RFP or the RFP vector control and the different N18.GFP domains were transiently expressed in JTAg cells, and their localization was analyzed (Fig. 3, A and B, and Fig. S3A). Interestingly, and in line with a recent report (56), ectopic expression of Unc119a.RFP resulted in retargeting of LckN18.GFP to the TGN in a manner reminiscent of Nef (70% ± 6.2% versus 10% ± 2.1% for the RFP control). LynN18.GFP (71.2% ± 5.1%), SrcN18.GFP (70.8% ± 6.2%), HaspbN18.GFP (68.3% ± 4.2%), HckN18.GFP (64.4% ± 6.1%), and FgrN18.GFP (61.2% ± 4.3%) were also retargeted to the TGN by Unc119a.RFP with similar efficiency. In contrast, the subcellular distribution of FynN18.GFP (20.4% ± 2.3%) and YesN18.GFP (17.5% ± 2.7%) was unaffected by ectopic expression of the LPC.

We next sought to assess the consequences of deleting exogenous Unc119a on SH4 domain localization. However, only moderate changes were observed in Unc119a expression and LckN18.GFP localization upon treatment of JTAg cells with siRNA oligos specific for Unc119a (Fig. S4, A–C). Because PDE6δ also acts as an LPC, and exogenous expression resulted in TGN retargeting of a similar although not identical subset of SFK SH4 domains to the TGN (data not shown), cells were treated simultaneously with siRNA oligos specific for Unc119a and PDE6δ. In line with a recent report on intracellular accumulation of the SFK Src, but not Fyn, that combined knock-down of Unc119a and PDE6δ in fibroblasts (56), simultaneous treatment with siRNA oligos targeting Unc119a and PDE6δ reduced expression of both LPCs in JTAg cells (Fig. S4, C and E). This was paralleled by LckN18.GFP retargeting to the TGN (72.3% ± 1.9% compared with 20.1% ± 2.5% in control cells) (Fig. 3, C and D, and Fig. S3B). LynN18.GFP was retargeted to
the TGN by Unc119a and PDE6δ knockdown with a similar efficiency as LckN18.GFP (72.6% ± 1.6%). However, the localization of FynN18.GFP (21.2% ± 2.3% retargeted) and YesN18.GFP (21.7% ± 2.7% retargeted) was not affected by combined knockdown of Unc119a and PDE6δ.

Similar results were obtained when Arl3.CFP, the GTPase that binds to Unc119a for cargo release (55, 64), was transiently expressed in JTAg cells along with the panel of N18.GFP SH4 domains. Ectopic expression of Arl3.CFP (Fig. 4, A and B, and Fig. S5A) caused retargeting to the TGN for LckN18.GFP (80.2% ± 2.2%), LynN18.GFP (60.6% ± 2.3%), SrcN18.GFP (63.6% ± 4.2%), HaspN18.GFP (78.5% ± 7.9%), and HckN18.GFP (84.3% ± 2.3%), whereas the distribution of FgrN18.GFP (25.5% ± 4.6%), FynN18.GFP (19.8% ± 4.3%), and YesN18.GFP (18.0% ± 1.3%) remained unchanged by the presence of Arl3.CFP. Retargeting by Arl3.CFP was also observed for LckN18.RFP (Fig. S5B). Again, treatment with siArl3 alone had only partial effects on the localization of Lck (Fig. S4, A–C). We tested the role of the related GTPase Arl2, which is also able to release cargo from Unc119a (55, 64), and found that overexpression of Arl2 caused retargeting of a similar, although not identical, subset of SFK SH4 domains compared with Arl3 (data not shown). Simultaneous knockdown of both GTPases led to TGN accumulation of LckN18.GFP (71.8% ± 0.8% retargeted compared with 21.4% ± 1.8% in the control) and LynN18.GFP (72.1% ± 1.2% retargeted) but not of FynN18.GFP (22.5% ± 1.6% retargeted) and YesN18.GFP (18.4% ± 1.8% retargeted) (Fig. 4, C and D, and Figs. S4, D and E, and S5C). Overexpression of GTPases or LPCs involved in SH4 domain transport thus phenocopies targeting of these components by siRNA, suggesting that overexpression of these components exerts dominant-negative effects. Together, these results revealed an intriguing overlap in the spectrum of SH4 domains that are specifically retargeted to the TGN by ectopic expression of Nef or manip-
ulation of expression levels of LPC and GTPase transport components (Table S1).

TGN accumulation by Unc119a does not require physical association of the LPC with the SH4 domain as revealed by co-immunoprecipitation

As Unc119a binds lipid moieties in a hydrophobic binding pocket (51), we wanted to know whether retargeting of the SH4 domains by Unc119a was the result of such physical association. Unc119a was readily co-immunoprecipitated with LckN18.GFP following GFP-trap isolation from transiently transfected JTAg cell lysates (Fig. 5A). This interaction was specific, as neither LckN18.GFP mutants in which one (C3S or C5S) or both (C3S,C5S) of the palmitoylated cysteine residues that are important for membrane association of the SFK were mutated to serine nor the RFP control were found to associate with LckN18.GFP. We next wanted to map the determinants in Unc119a for association with LckN18 and created a set of Unc119a mutants that are defective in individual binding domains. Mutations that disrupt the SH2-interacting domain and the periphery of the lipid binding pocket (Y194A) or the SH3-interacting domain (R56A, P59A, and P62A) (78) did not affect the efficiency of co-isolation of Unc119a.RFP with LckN18.GFP (Fig. 5B). In contrast, disrupting the central residue of the lipid-binding pocket that makes contact with the fatty acid (Y131A) (64) abrogated the association of the SH4 domain with the LPC. The association detected by this co-immunoprecipitation approach thus likely reflects direct binding of the Lck SH4 domain fatty acids to the hydrophobic pocket of Unc119a.

We next tested whether physical association between the LPC and the Lck SH4 domain detected by this co-immunoprecipitation approach correlates with retargeting of the SH4 domain to the TGN. Transient expression of Unc119a mutants in JTAg cells revealed that individual mutations of Unc119a interaction motifs with fatty acids, SH2 domains, or SH3 domains did not impair their ability to cause TGN retargeting of LckN18.GFP (Fig. 5C and Fig. S6). The ability of Unc119a to...
co-immunoprecipitate with the Lck SH4 domain is therefore dispensable for the retargeting effect. This also suggests that retargeting of LckN18.GFP to the TGN upon ectopic expression of Unc119a likely reflects a dominant-negative effect on SH4 domain transport because of sequestration of Unc119a ligands essential for transport. Moreover, screening of our panel of SH4 domains revealed that only the SH4 domain of Lck, but not those of Src, Haspb, Hck, Fgr, Fyn, or Yes, associates with Unc119a with sufficient affinity for detection by our co-immunoprecipitation approach (Fig. S7A). We conclude that physical association with Unc119a, as detected by co-immunoprecipitation, does not define the sensitivity of SH4 domains for TGN retargeting by Nef or LPC transport machinery.

**A single amino acid determines Lyn SH4 domain sensitivity for TGN retargeting by Nef**

We next sought to map an SH4 domain signature that governs its sensitivity to TGN retargeting by Nef. Initial attempts to achieve this based on the comparison of Nef-sensitive Lck and Nef-insensitive Fyn SH4 domains were hampered by the low degree of sequence conservation between these two SH4 domains (Fig. 6A). In contrast, Lyn and Yes have a very similar SH4 domain amino acid sequence, although the SH4 domain of Lyn is retargeted by Nef and the Yes SH4 domain is not. To identify the determinant for Nef sensitivity in the Lyn SH4 domain, we first generated two chimeric constructs between residues 1–10 and 11–18, LynYesN18 and YesLynN18 (Fig. 6, B and C, and Fig. S7A). Interestingly, following transient expression in JTAg cells, LynYesN18.GFP was retargeted to the TGN by Nef (65.6% ± 7.8%) with similar efficiency as LynN18.GFP. In contrast, relocalization of YesLynN18.GFP was not affected by Nef (15.6% ± 1.2%), akin to YesN18.GFP. The key determinant for sensitivity for retargeting by Nef is therefore present in the first 10 amino acids of the SH4 domain.

---

**Figure 5. Lck SH4 domain fatty acids directly interact with the Unc119a hydrophobic binding pocket.** A, GFP-trap isolation was carried out with LckN18.GFP WT or the indicated LckN18.GFP single (C3S or C5S) or double (C3S,C5S) palmitoylated cysteine-to-serine mutants. The co-immunoprecipitation assay was examined by Western blot analysis. Anti-RFP antibody probed for input (second panel) and bound (first panel) RFP or Unc119a.RFP that co-immunoprecipitated with N18.GFP. Anti-GFP antibody probed for bound (third panel) or input (fourth panel) LckN18.GFP. B, association of LckN18.GFP with Unc119a.RFP or its lipid binding domain (Y131A), SH2- (Y194A), or SH3-interacting domain (R56A, P59A, and P62A) mutants was assayed by GFP-trap isolation of LckN18.GFP followed by Western blot analysis. Input GFP or LckN18.GFP is shown in the fourth panel (α-GFP), and bound GFP or LckN18.GFP is shown in the third panel (α-RFP). Input (second panel) or bound (first panel) RFP or Unc119a.RFP WT or binding mutants that were co-immunoprecipitated were analyzed by Western blot analysis, probing with an antibody against RFP (α-RFP). C, LckN18.GFP and Unc119a.RFP or the indicated interacting domain mutants were transiently expressed in JTAg cells. Representative confocal images of LckN18.GFP and the corresponding Unc119a.RFP are shown. Arrowheads represent retargeting of LckN18.GFP. Scale bar = 2 μm. D, GFP-trap isolation was done with the indicated SH4.GFP domains. Western blot analysis was carried out and assayed for bound or input N18.GFP (third and fourth panels, α-GFP, respectively) and input (second panel, α-RFP) or bound (first panel, α-RFP) RFP or Unc119a.RFP.
negatively charged glutamic acid for Yes. Replacing glycine 8 with a glutamic acid in LynN18 (LynN18 (G8E)) markedly reduced TGN retargeting by Nef (20.3%/H11006 7.2% retargeted). This was also seen in primary T cells, where LynN18 (G8E).GFP was fully insensitive to Nef-mediated retargeting (8.0%/H11006 3.8%) (Fig. 6, D and E, and Fig. S7 B). The G8E mutation abrogated retargeting of LynN18.GFP by Nef variants from HIV-1 (NL4.3, 21.1%+/H11006 0.5%), HIV-2 (Cbl23, 21.7%+/H11006 0.9%), and SIV (mac239, 22.9%+/H11006 5.3%) (Fig. 6, F and G, and Fig. S7 C). Furthermore, this single amino acid mutation also strongly reduced TGN retargeting of LynN18.GFP by Unc119a overexpression (30.2%+/H11006 4.7%), knockdown of Unc119a/PDE66 (21.3%+/H11006 1.8%) (Fig. 7, A–D, and Fig. S8, A and B), overexpression of Arl3.CFP (17.4%+/H11006 2.6%), or knockdown of Arl3/Arl2 (20.5%+/H11006 1.9%) (Fig. 7, E–H, and Fig. S8, C and D). These results demonstrate that the sensitivity of the Lyn SH4 domains for retargeting by Nef can be determined by a single amino acid. Analogous analysis of additional SH4 domains was hampered by the loss of overall membrane association upon introduction of the respective amino acid changes (Table S2).

Prevention of SH4 domain DRM association is dispensable for TGN targeting by Nef

Our previous results showed that Nef significantly reduces DRM microdomain incorporation of full-length Lck and LckN18 but not of Fyn (47). We therefore tested whether TGN retargeting is associated with reduced DRM incorporation for all Nef-sensitive SH4 domains. To this end, DRM microdomain association of LckN18.GFP, FynN18.GFP, LynN18.GFP, and LynN18 (G8E).GFP was analyzed in the absence or presence of Nef by membrane flotation following cell lysis in cold Triton X-100 detergent (Fig. 8) (47, 79). Cell lysate prior to flotation (input) as well as DRM and soluble fractions of transiently transfected JTAg cells were investigated by Western blot analysis. Transferrin receptor and linker of activated T cells (LAT) served as markers for the soluble and DRM fractions, respec-
Block of SH4 domain anterograde transport by HIV-1 Nef

For all four N18.GFP proteins analyzed, at least 20% of all protein was associated with the DRM fraction in the absence of Nef (see Fig. 8E for quantification). As expected, co-expression of Nef significantly reduced DRM association of LckN18.GFP (6.7% ± 4.8% of DRM associated; Fig. 8A and E), and DRM association of FynN18.GFP was unaffected by Nef (24.4% ± 10.8% of DRM associated; Fig. 8, B and E). DRM association of LynN18.GFP (33.9% ± 3.8% versus 25% ± 10.4% of DRM associated) and LynN18 (G8E).GFP (26.4% ± 3.7% versus 18.5% ± 6.2% of DRM associated) was also
comparable in the absence or presence of Nef, respectively (Fig. 8, C–E), although Nef efficiently retargets LynN18.GFP to the TGN (Fig. 1, C and D). DRM exclusion is therefore not a requirement for Nef-mediated retargeting of SH4 domains to the TGN.

**Nef inhibits anterograde transport of SH4 domains**

Because the sensitivity of SH4 domains for TGN retargeting by Nef could not be explained by their DRM association pattern, we next sought to address whether Nef affects the same anterograde transport step of all sensitive SH4 domains. To follow the transport of SH4 domains from biosynthesis to steady-state distribution, we adopted a previously established plasmid microinjection approach (80, 81). HT1080 cells were microinjected with expression plasmids for LckN18.GFP and Nef.RFP or RFP control, and subcellular localization of N18.GFP was analyzed at various time points after microinjection relative to the TGN marker TGN46 (Fig. 9A and Fig. S9A, see Fig. 9B for quantification). With this approach, newly synthesized proteins could be detected as early as 30 min post-microinjection and at 2 h post-microinjection (p.m.), a sufficient number of cells displayed robust protein expression for analysis. When expressed with the RFP control, LckN18.GFP was initially observed predominantly at a perinuclear compartment that significantly overlapped with TGN46 (detectable at the PM in 18.6% ± 5.9% of cells). Subsequently, anterograde transport delivered LckN18.GFP to the PM, resulting in a majority of cells in which LckN18.GFP was detectable at the PM 6 h p.m. (detectable at the PM in 83.7% ± 10.8% of cells). 24 h p.m., subcellular distribution of LckN18.GFP reached its steady state with localization at the PM (detectable at the PM in 97.4% ± 8.6% of cells), the cytoplasm, and the TGN (Fig. 9A).

When expressed with Nef.RFP, LckN18.GFP localized to the TGN at 2 h p.m., similar to the scenario in RFP-expressing cells (detectable at the PM in 14.6% ± 8.5% of cells). However, at subsequent time points, this subcellular localization was preserved, and no significant delivery to the PM was observed (detectable at the PM in 13.2% ± 7.1% and 15.2% ± 8.8% of cells at 6 and 24 h p.m., respectively). Nef thus inhibits Lck SH4 domain anterograde transport in HT1080 cells in a similar manner to PM delivery of full-length Lck in fibroblasts and T cells (19, 26–28, 56). We then tested whether the sensitivity of SH4 domains for TGN retargeting by Nef is associated with such a block in anterograde transport (Fig. 9, C and D, and Fig. S9B). Nef-insensitive FynN18.GFP was delivered more rapidly to the PM than LckN18.GFP in control cells (already detected at the PM at 2 h p.m. in 80.2% ± 4.1% of cells), and the presence of Nef did not affect PM delivery (PM-localized in 81.2% ± 3.4% of cells). The PM delivery kinetics of Nef-sensitive LynN18.GFP in the presence of RFP more closely resembled that of LckN18.GFP, with completion at 6 h p.m. (PM localized in 80.1% ± 6.2% of cells). The presence of Nef.RFP resulted in marked reduction of PM delivery (PM localized in 22.7% ± 3.2% of cells at 6 h p.m.), and LynN18.GFP was instead associated with a TGN46-positive compartment. In contrast, the Nef-insensitive mutant LynN18 (G8E) displayed PM delivery kinetics similar to FynN18.GFP (PM localized in 81.2% ± 3.4% of cells). The PM delivery kinetics of Nef-sensitive LynN18.GFP in the presence of RFP more closely resembled that of LckN18.GFP, with completion at 6 h p.m. (PM localized in 80.1% ± 6.2% of cells). The presence of Nef.RFP resulted in marked reduction of PM delivery (PM localized in 22.7% ± 3.2% of cells at 6 h p.m.), and LynN18.GFP was instead associated with a TGN46-positive compartment. In contrast, the Nef-insensitive mutant LynN18 (G8E) displayed PM delivery kinetics similar to FynN18.GFP (PM localized in 79.9% ± 3.0% at 2 h p.m.) and was not observed at intracellular membranes before reaching the PM, and the presence of Nef.RFP had no effect.
Figure 9. SH4 domain sensitivity to TGN retargeting by Nef is determined at the level of anterograde transport. A, HT1080 cells were microinjected with LckN18.GFP and RFP or Nef.RFP expression plasmids and harvested at 2, 6, or 24 h post-microinjection, as indicated. Cells were then stained with anti-TGN46 antibody as a TGN marker. Shown are representative confocal images. Accumulation at the TGN is indicated by the arrowheads, and N18.GFP localization at the PM is marked by the arrows. The cell periphery is indicated by the dotted line for the cells in which the PM is not clearly visible. Scale bar = 5 μm. B, the percentage of cells with LckN18.GFP at the PM is shown as the mean of three independent experiments ± S.D. The percentage of LckN18 at the PM is compared between LckN18 microinjected with RFP (black columns) or with Nef.RFP (white columns). C, HT1080 cells were microinjected with FynN18.GFP, LynN18.GFP, or LynN18 (G8E).GFP with RFP or Nef.RFP expression plasmids and harvested at 2 or 6 h post-microinjection. Shown are representative confocal images of the N18.GFP construct at 6 h post-microinjection. N18.GFP retargeting to the TGN is indicated by the arrowhead, whereas N18.GFP localization at the PM is marked with arrows. The cell periphery is indicated by the dotted line for the cells in which the PM is not clearly visible. Scale bar = 5 μm. D, the percentage of cells with FynN18.GFP, LynN18.GFP, or LynN18 (G8E).GFP at the PM is given as the mean of three independent experiments ± S.D. The percent of N18.GFP at the PM is compared for RFP (black columns) and Nef.RFP (white columns).

Discussion

The HIV-1 pathogenesis factor Nef inhibits anterograde transport of the SFK Lck, but not the closely related Fyn, to disrupt early TCR signaling (19, 26–29, 47). The effects of Nef on SFK transport are recapitulated by the 18-amino acid SFK SH4 domain, but the molecular basis for the selectivity of Nef for affecting SH4 domain transport was unknown. The goal of this study, therefore, was to define the parameters that govern the responsiveness of SH4 domains to Nef and to assess the involvement of LPC transport machinery in the anterograde transport of Nef-sensitive SH4 domains. Our analyses revealed that SFK SH4 domains generally segregate into Nef-sensitive and Nef-resistant groups that are retargeted to the TGN or remain unaffected by the presence of the viral protein, respectively. With position 8, a single amino acid was identified as an essential determinant for Nef sensitivity of the Lyn SH4 domain. For Nef-sensitive SH4 domains, ectopic expression of the LPCs Unc119a or PDE6β or the GTPase Arl3 or Arl2 or simultaneous silencing of endogenous expression of both LPCs or GTPases phenocopied the effect of Nef. This suggests that anterograde transport of these SH4 domains relies on these components and that several LPCs and GTPases can exert this function. TGN retargeting of SH4 domains did not require a Nef-induced reduction in DRM association but did correlate with a block of transport toward the PM at the level of the TGN and with the use of a slower PM delivery route than that used by Nef-insensitive SH4 domains. Together, these results suggest that Nef interferes with an anterograde LPC/GTPase transport pathway that is used for PM delivery by a selective set of SH4 domains.

Although TGN retargeting of Lck and its functional implication in T cell activation and HIV replication have long been recognized (18, 19, 26), little was known about Lck transport to the PM and how Nef affects the subcellular distribution of the SFK. Our results confirm and extend the observation that Nef affects an anterograde transport pathway by which some, but not all, SH4 domains are delivered to the PM. Consistent with the observed solubilization of Lck in the cytoplasm in the presence of Nef by ectopic expression of Unc119a (27), our results strongly suggest that, analogous to K-Ras and PDE6β (59, 60), Nef-sensitive SH4 domains share a nonclassical transport pathway in which GTPase-mediated release of the solubilized LPC-bound SH4 domain drives insertion into anterograde transport platforms destined for the PM (see Fig. S10 for a schematic model). Of note, during the final stages of preparing this manuscript, Konitsotis et al. (56) reported direct evidence for such a model by demonstrating that, in fibroblasts, PM delivery of SFKs such as Src depends on solubilization by Unc119a. According to this model, disruption of active anterograde transport at any step results in rapid accumulation of the SFK at
Block of SH4 domain anterograde transport by HIV-1 Nef

the TGN because of an abundance of negative charge in the endomembrane system. This likely explains why depletion (41, 56) and overexpression of essential components of the LPC transport machinery (27, 56) result in comparable retargeting of the SFK to the TGN.

In line with previous comparisons between Lck and Fyn (47), our screen of SH4 domains for sensitivity to TGN retargeting by HIV-1 Nef revealed that fundamental differences exist in the mechanisms by which individual SH4 domains are transported to the PM. Although PM delivery of Nef-sensitive Lck, Lyn, Src, Haspb, and Hck appears to rely on Unc119a and Arl3, Nef-insensitive Fgr, Fyn, and Yes employ a PM delivery pathway that is independent of this LPC transport machinery (Fig. S10). This likely reflects that Nef-insensitive SH4 domains are rapidly transported to the PM following biosynthesis without appreciable transport intermediates at intracellular membranes and, thus, without requirement for LPC machinery. In contrast, PM delivery of Nef-sensitive SH4 domains is slower and occurs subsequent to initial targeting to Golgi membranes (Fig. 9 and data not shown), explaining the necessity for protein solubilization. In this scenario, intrinsic properties of the SH4 domain determine, concomitant to or immediately after biosynthesis, whether this SFK is cargo of the Nef-sensitive or -insensitive pathway. Of note, we were able to shift the Lyn SH4 domain from the Nef-sensitive to the Nef-insensitive PM delivery pathway by a single amino acid change. Because the amino acid exchange from a glycine to a glutamate considerably altered the charge of the SH4 domain, we speculate that the affinity of SH4 domains to membranes with specific lipid composition, charge, and/or curvature or to individual LPCs dictates by which transport pathway PM delivery occurs. Depending on the nature of the SH4 domain, a certain flexibility in the LPC and GTPase mediating PM delivery of Nef-sensitive SH4 domains seems to exist, as ectopic expression of PDE6β or Arl2 caused SH4 retargeting of some but not all Nef-sensitive SH4 domains, whereas LPCs Unc119b or HO2 had no effect on any of the SH4 domains tested (data not shown). Differences in, e.g., expression levels of individual components of this transport machinery may result in the usage of cell type–specific combinations of these components. This could explain why Unc119a mediates Fyn PM delivery in fibroblasts (56) but not in CD4 T cells (this study).

Although we did not identify the precise molecular mechanism by which Nef disrupts PM delivery and causes TGN targeting of specific SFKs, our results provide the first mechanistic insight. In principle, Nef may affect any step of the LPC transport cycle, from SH4 domain solubilization by the LPC to release into transport competent membrane platforms or their forward transport. Because Nef expression results in the accumulation of membrane-associated SH4 domains at the level of the TGN rather than as soluble cytoplasmic SH4 domains and affects SH4 domains with (Lck, Hck, Lyn, Haspb) or without palmitoylation (Src), Nef does not seem to act by altering the steady-state SH4 domain acylation pattern. Initially thought to be a correlate for the activity of Nef in this process, exclusion of the SH4 domain from DRM transport platforms was dispensable for Nef-mediated retargeting to the TGN. This suggests that Nef acts to inhibit anterograde transport at a step after incorporation of the SH4 domain into specific membrane microdomains and that the loss of this association for some Nef-sensitive SH4 domains may be a secondary consequence of transport inhibition. Based on the shared use as transport machinery for PM delivery of Nef-sensitive SH4 domains, Unc119a and Arl3 could be potential targets of inhibition by Nef. However, the fact that Fgr SH4 domain transport relies on Unc119a, although PM delivery is not affected by Nef (Fig. 3B), suggests that Nef does not target Unc119a directly. Finally, interference with Arl3 activity would be expected to stabilize soluble complexes of the LPC and SH4 domain, which is not observed in cells expressing Nef. Together, our results suggest that Nef prevents the initial loading of TGN-resident SH4 domains on the LPC and/or blocks forward transport of vesicles with their SH4 domain cargo. Considering that PM delivery of SH4 domains is thought to occur via recycling endosomes (56, 82, 83) and that Nef can interfere with trafficking of recycling endosomes (10, 84, 85), the latter hypothesis is particularly attractive. Such a mechanism could explain the effects of Nef on PM delivery of peripheral membrane proteins as well as transmembrane receptors.

This study emphasizes that SFKs are delivered to the PM by sophisticated nonclassical transport pathways, one of which is subject to potent inhibition by the HIV pathogenesis factor Nef. Thus, HIV-1 Nef hijacks SFK PM delivery mediated by the LPC Unc119a and the GTPase Arl3 to adjust T cell activation for optimized virus replication. In addition, the HIV structural protein Gag was recently shown to be delivered to the PM via the myristate-binding protein HO2, acting as an LPC. This facilitates evasion of viral replication intermediates from innate immune recognition and biogenesis of new viral particles (57). LPC-mediated transport thus emerges as a central cellular pathway exploited by HIV to create an optimal cellular environment for virus spread. Viral proteins that utilize or disturb this pathway will continue to serve as valuable tools for the dissection of these cellular transport pathways.

Experimental procedures

Cells and reagents

JTAg cells were grown in RMPI 1640 medium supplemented with 10% FCS and 1% penicillin-streptomycin (Invitrogen). HT1080 cells were cultivated in DMEM supplemented with 10% FCS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 10 mM HEPES (pH 7.4). Primary T cells were purified as described previously (86) and cultured in RMPI 1640 medium supplemented with 10% FCS and 1% penicillin-streptomycin.

The following primary antibodies were used: sheep anti-TGN46 (Bio-Rad), mouse anti-Myc (Sigma-Aldrich), rat anti-GFP (Chromotek), rabbit anti-RFP, mouse anti-transferrin receptor (Life Technologies), rabbit anti-LAT (Cell Signaling Technology), rabbit anti-Arl3 (Proteintech), rabbit anti-Unc119a (Thermo Fisher Scientific), and mouse anti-Lck (Santa Cruz Biotechnology). Secondary goat anti-rabbit, goat anti-mouse, anti-sheep Alexa Fluor 568, anti-mouse Alexa Fluor 350, and anti-sheep Alexa Fluor 350 were all purchased from Invitrogen.
Expression plasmids

SH4 domain plasmids were cloned into the pEGFP.N1 or pERFP.N1 expression vector as indicated. Cloning was carried out using annealed oligos that corresponded to the sequence of the N-terminal 54 nucleotides coding for the 18 amino acids of the SH4 domain. The oligos were designed to be compatible with the BamHI and Nhel double-digested pEGFP.N1 vector for subsequent ligation. The LynN18 (G8E).GFP mutant was cloned in the same way, with the corresponding mutation included in the oligos. The GenBank accession numbers of the Src family kinase sequences are as follows: Lyn, M16038; Src, NM_198291; Haspb, XM_001683386; Hck, NM_001172133; Lck, X13529; Fgr, NM_002037; Fyn, NM_002037; Yes, NM_005433.

Unc119a.RFP was described previously (27). The lipid binding pocket and SH2-interacting domain mutant (Y194A), the SH3-interacting domain mutants (R56a, P59a, and P62a), and the lipid binding domain mutant (Y131A) were generated by site-directed mutagenesis. Arl3.CFP was subcloned into the pECFP.N1 vector from the pcDNA3.1 expression vector. Arl3.pcDNA3.1 was kindly provided to us by Dr. Richard Kahn (87). The Nef.YFP alleles were described previously (88).

Transfection of cells

JTAg cells (1 × 10⁷) were transfected with 15–30 µg of total plasmid DNA via electroporation (950 microfarad, 250 V, Bio-Rad GenePulser) as described previously (49). Primary T cells were nucleofected with N18.GFP (2 µg) and RFP or Nef.RFP (2 µg) using the Amaxa Human T Cell Nucleofector Kit (Lonza) as described previously (89).

JTAg cells (1 × 10⁷) were transfected with siRNA via electroporation (950 microfarad, 250 V, Bio-Rad GenePulser). Cells were electroporated with 250 pmol or 500 pmol of siRNA against Unc119a or Arl3. Alternatively, they were electroporated with 250 pmol (500 pmol total) or 500 pmol (1000 pmol total) each Unc119a and PDE6δ or Arl3 and Arl2. Cells were collected at 24 h or 48 h post-electroporation for analysis. JTAg cells co-expressing N18.GFP were electroporated with N18.GFP plasmid DNA (10 µg) in tandem with 500 pmol of total siRNA and collected for analysis at 24 h.

Immunofluorescence analysis and quantification

Glass microscope coverslips were incubated with 0.01% poly-l-lysine (Sigma-Aldrich) for 10 min at room temperature. JTAg cells (3 × 10⁵) were plated on the coverslips, incubated for 5 min, and fixed for 10 min with 3% paraformaldehyde in PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 1 min and blocked for 30 min with 1% BSA in PBS. Cells were then incubated with primary antibody (anti-TGN46, 1:300; anti-Myc, 1:400; anti-Lck, 1:50) for 1 h at room temperature and fluorochrome-labeled secondary antibody (1:2000) for 1 h at room temperature. Coverslips were then mounted on slides with Mowiol. Based on a previously established workflow (27, 47, 70), 100 cells for each condition and three independent experiments were evaluated by wide-field microscopy. N18.GFP or endogenous Lck localization was determined to be predominantly at the plasma membrane or at distinct intracellular membranes, i.e. the TGN, as established previously by pixel quantification of reconstituted z-stacks (27). By this analysis, ~75% of total Lck signal per cell was found in the area of the TGN in cells in which TGN targeting of Lck occurs, compared with ~18% of total Lck signal in this area in cells without apparent Nef-mediated retargeting. Results are then presented as mean values and standard deviation for at least three independent experiments. Graphs were first made in Excel and imported into Illustrator. Confocal images were acquired using a Leica TCS SP5 confocal microscope with a ×100 (JTAg cells) or ×63 (HT1080 cells) Plan-Apo objective lens. Single-plane images were taken with Leica LAS AF (Leica Application Suite for Advanced Fluorescence) software. Images were minimally processed with Photoshop CC to adjust the overall brightness and contrast of the image, applying identical thresholds to all images.

Quantitative PCR

Analysis was carried out as described previously (90). Briefly, total RNA was extracted using the Nucleospin RNA II Kit (Macherey-Nagel). It was then reverse-transcribed using the SuperScript One-Step RT-PCR system (Life Technologies) according to the manufacturer’s protocol. Reactions were then performed on an ABI Prism 7500 sequence detection system (Applied Biosciences). The program used was as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used for normalization. The following primers were used: Unc119a forward, 5’-TTTGTCCGCTACCACTCAC-3’; Unc119a reverse, 5’-ATGCAGACTTTGACAGG-3’; PDE6δ forward, 5’-TCAAGTGCAAGCAGTGC-3’; PDE6δ reverse, 5’-TGGAGTTAGGGAATACAAAGCC-3’; Arl3 forward, 5’-GCCCTGAAATTTGCAGAAGC-3’; Arl3 reverse, 5’-TTTGCAAGCACCTTGATGC-3’; Arl2 forward, 5’-ACCTGCGGAATCTTTGAGAG-3’; Arl2 reverse, 5’-TAGCAGATGAGGGTTGC-3’.

Western blot analysis

Proteins were separated by 12% SDS-PAGE and electroblotted to nitrocellulose membranes. Membranes were blocked in 5% milk in PBS-T for 30 min at room temperature and probed with the indicated primary antibody (1:1000 in 3% BSA in PBS-T) overnight at 4 °C. Membranes probed with anti-Unc119a (1:300 in 1% BSA in PBS-T) were first blocked in 5% milk in 1% BSA. After washes with PBS-T, the membrane was incubated with the corresponding horseradish peroxidase secondary antibody (1:10,000 in PBS-T) for 1 h at room temperature and again washed with PBS-T. Protein bands were visualized using ECL chemiluminescence substrate (Thermo Scientific). Band intensity was analyzed using Image J.

GFP immunoprecipitation binding assays

JTAg cells were transfected with SH4.GFP (15 µg) and RFP or Unc119a.RFP (15 µg). 24 h after transfection, 1 × 10⁷ cells were collected for each immunoprecipitation assay and washed with PBS prior to lysis in 200 µl of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 75 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, 0.4% NP-40, and protease inhibitors (Sigma)). Cells were then incubated on ice for 30 min with intermittent
pipetting. Cells were then spun for 10 min at 6000 rpm and 4 °C, and 20 μl was removed as the input. 50 μl of GFP-Trap beads (ChromoTek) were washed three times with cold PBS before being incubated with the remaining supernatant from the lysed cells. The beads and supernatant were then incubated for 2 h at 4 °C with constant gentle end-over-end mixing. The beads and supernatant mix were then spun for 2 min at 3000 rpm at 4 °C, and the supernatant was removed. The beads were washed five times with lysis buffer, resuspended in 100 μl of 2× sample buffer, and examined by Western blot analysis.

**Detergent-resistant membrane assay**

DRM assays were carried out as described previously (47) with a few alterations. Briefly, JTAγ cells were transfected with SH4.GFP (15 μg) and RFP or Nef.RFP (15 μg) plasmids. 24 h post-transfection, cells were collected, lysed in 500 μl of ice-cold TXNE buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Sigma)), and incubated on ice for 20 min. Cells were then homogenized by passage through a pipette tip 15 times, adjusted to 37.5% Optiprep (Life Technologies), and transferred to a SW60 centrifuge tube. After centrifugation, 500-μl fractions were collected from the top, with fraction 2 corresponding to the DRM fraction and fractions 7 and 8 to the soluble fractions. The input and fractions 2, 7, and 8 were assayed by Western blot analysis.

**Microinjection**

Anterograde transport was analyzed using microinjection assays as described previously (80,81). Briefly, 2 × 10^5 HT1080 cells were seeded on coverslips 24 h prior to microinjection. Nuclei of HT1080 cells were microinjected using an AIS-2 microinjection apparatus with pulled borosilicate glass capillaries. Prior to microinjection, plasmids encoding SH4.GFP (30 ng/μl final concentration) and RFP or Nef.RFP (30 ng/μl final concentration) were mixed. After microinjection, cells were incubated at 37 °C for 2, 4, 6, or 24 h, as indicated, and fixed with 4% paraformaldehyde in PBS. Cells were then permeabilized with 0.1% Triton-X-100 for 1 min, blocked, and stained with sheep anti-TGN46 (1:300) for 1 h at room temperature. After washing with PBS, cells were stained with anti-sheep Alexa Fluor antibody (1:2000, Invitrogen). For quantification, the percentage of cells with the SH4 domain clearly at the PM was determined. For each time point, 100 cells were counted for three independent experiments, and the mean and standard deviation is given.

**Author contributions**—A. J. C. and R. W. data curation; A. J. C. and O. T. F. formal analysis; A. J. C. and O. T. F. writing-original draft; A. J. C. and O. T. F. writing-review and editing; O. T. F. conceptualization.

**Acknowledgments**—We thank Miriam Geist for guidance and training in the initial phase of this project and Quan Chanthalengsy and Nadine Tibroni for technical assistance. We are grateful for the kind gift of plasmids from Dr. Richard A. Kahn.

**References**

1. Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C., Lawson, V. A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., et al. (1995) Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science 270, 988–991 CrossRef Medline

2. Kestler, H. W., 3rd, Ringer, D. I., Mori, K., Panici, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C. (1991) Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65, 651–662 CrossRef Medline

3. Kirchhoff, F., Greenough, T. C., Brltretter, D. B., Sullivan, J. L., and Desro- siers, R. C. (1995) Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. 332, 228–232 CrossRef Medline

4. Geyer, M., Fackler, O. T., and Peterlin, B. M. (2001) Structure–function relationships in HIV-1 Nef. EMBO Rep. 2, 580–585 CrossRef Medline

5. Kirchhoff, F., Schindler, M., Specht, A., Arhel, N., and Münch, J. (2008) Role of Nef in primate lentiviral immunopathogenesis. Cell. Mol. Life Sci. 65, 2621–2636 CrossRef Medline

6. Garcia, J. V., and Miller, A. D. (1991) Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 350, 508–511 CrossRef Medline

7. Schwartz, O., Maréchal, V., Le Gall, S., Lemmonier, F., and Heard, J. M. (1996) Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. 2, 338–342 CrossRef Medline

8. Stumptner-Cuvelette, P., Morchoisne, S., Dugast, M., Le Gall, S., Raposo, G., Schwartz, O., and Benaroch, P. (2001) HIV-1 Nef impairs MHC class II antigen presentation and surface expression. Proc. Natl. Acad. Sci. U.S.A. 98, 12144–12149 CrossRef Medline

9. Michel, N., Allespach, I., Venzke, S., Fackler, O. T., and Keppler, O. T. (2005) The Nef protein of human immunodeficiency virus establishes superinfection immunity by a dual strategy to downregulate cell-surface CCR5 and CD4. Curr. Biol. 15, 714–723 CrossRef Medline

10. Chaudhry, A., Das, S. R., Jameel, S., George, A., Bal, V., Mayor, S., and Rath, S. (2008) HIV-1 Nef induces a Rab11-dependent routing of endocytosed immune costimulatory proteins CD80 and CD86 to the Golgi. Traffic 9, 1925–1935 CrossRef Medline

11. Haller, C., Müller, B., Fritz, J. V., Lamas-Murua, M., Stolp, B., Pujol, F. M., Keppler, O. T., and Fackler, O. T. (2014) HIV-1 Nef and Vpu are functionally redundant broad-spectrum modulators of cell surface receptors, including tetraspanins. J. Virol. 88, 14241–14257 CrossRef Medline

12. Galaksi, J., Ahmad, F., Tibroni, N., Pujol, F. M., Müller, B., Schmidt, R. E., and Fackler, O. T. (2016) Cell surface downregulation of NK cell ligands by patient-derived HIV-1 Vpu and Nef alleles. J. Acquir. Immune Defic. Syndr. 72, 1–10 CrossRef Medline

13. Cerboni, C., Neri, F., Casartelli, N., Zingoni, A., Cosman, D., Rossi, P., Santoni, A., and Doria, M. (2007) Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. J. Gen. Virol. 88, 242–250 CrossRef Medline

14. Matusalis, G., Postel, M., Santoni, A., Cerboni, C., and Doria, M. (2012) The human immunodeficiency virus type 1 Nef and Vpu proteins downregulate the natural killer cell-activating ligand PVR. J. Virol. 86, 4496–4504 CrossRef Medline

15. Matheson, N. J., Sumner, J., Wals, K., Rapiteanu, R., Weekes, M. P., Vigan, R., Weinfel, J., Schindler, M., Antrobus, R., Costa, A. S., Frezza, C., Clish, C. B., Neil, S. J., and Lehner, P. J. (2015) Cell surface proteomic map of HIV infection reveals antagonism of amino acid metabolism by Vpu and Nef. Cell Host Microbe 18, 409–423 CrossRef Medline

16. Simmons, A., Aluvihare, V., and McMichael, A. (2001) Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. Immunity 14, 763–777 CrossRef Medline

17. Simmons, A., Gangadharan, B., Hodges, A., Sharrock, K., Prabhakar, S., García, A., Dwek, R., Zitzmann, N., and McMichael, A. (2005) Nef-mediated lipid raft exclusion of UbcH7 inhibits Cbl activity in T cells to positively regulate signaling. Immunity 23, 621–634 CrossRef Medline

18. Haller, C., Rauch, S., and Fackler, O. T. (2007) HIV-1 Nef employs two distinct mechanisms to modulate Lck subcellular localization and TCR induced actin remodeling. PLoS ONE 2, e1212 CrossRef Medline
19. Thoulouze, M. L., Sol-Foulou, N., Blanchet, F., Dautry-Varsat, A., Schwartz, O., and Alcover, A. (2006) Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. *Immunity* 24, 547–561 CrossRef Medline

20. Abraham, L., and Fackler, O. T. (2012) HIV-1 Nef: a multifaceted modulator of T cell receptor signaling. *Cell Commun. Signal.* 10, 39 CrossRef Medline

21. Fackler, O. T., and Baur, A. S. (2002) Live and let die: Nef functions beyond immunological synapse. *Mol. Biol. Cell.* 136, 1023–1035 CrossRef Medline

22. Xu, X. N., Laffert, B., Screaton, G. R., Kraft, M., Wolf, D., Kolanus, W., Stein, T., C, Ritzler, J., Barghorn, S., Kielian, M., Novembre, F. J., Peeters, M., Courgnau, V., Bailes, E., Roques, B., Sodora, D. L., Silvestri, G., Sharp, P. M., et al. (2006) Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1. *Cell 125*, 1055–1067 CrossRef Medline

23. Haller, C., Rauch, S., Michel, N., Hannemann, S., Lehmann, M. J., Kessler, O. T., and Fackler, O. T. (2006) The HIV-1 pathogenicity factor Nef interferes with maturation of stimulatory T-lymphocyte contacts by modulation of N-Wasp activity. *J. Biol. Chem.* 281, 19618–19630 CrossRef Medline

24. Pan, X., Rudolph, J. M., Abraham, L., Habermann, A., Haller, C., Krijnse-Locker, J., and Fackler, O. T. (2012) HIV-1 Nef compensates for disorganization of the immunological synapse by inducing trans-Golgi network-associated Lck signaling. *Blood 119*, 786–797 CrossRef Medline

25. Schindler, M., Münch, J., Kutsch, O., Li, H., Santiago, M. L., Bibollet-Ruche, F., Muller-Trutwin, M. C., Nombela, F., Peeters, M., Courgnau, V., Bailes, E., Roques, B., Sodora, D. L., Silvestri, G., Sharp, P. M., et al. (2006) Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1. *Cell 125*, 1055–1067 CrossRef Medline

26. Haller, C., Rauch, S., Michel, N., Hannemann, S., Lehmann, M. J., Kessler, O. T., and Fackler, O. T. (2006) The HIV-1 pathogenicity factor Nef interferes with maturation of stimulatory T-lymphocyte contacts by modulation of N-Wasp activity. *J. Biol. Chem.* 281, 19618–19630 CrossRef Medline

27. Pan, X., Rudolph, J. M., Abraham, L., Habermann, A., Haller, C., Krijnse-Locker, J., and Fackler, O. T. (2012) HIV-1 Nef compensates for disorganization of the immunological synapse by inducing trans-Golgi network-associated Lck signaling. *Blood 119*, 786–797 CrossRef Medline

28. Arhel, N. J., and Kirchhoff, F. (2009) Implications of Nef: host cell interactions in viral persistence and progression to AIDS. *Curr. Top. Microbiol. Immunol.* 339, 147–175 Medline

29. Salmon, R., J., Filby, A., Qureshi, I., Caserta, S., and Zamoyska, R. (2009) T-cell receptor proximal signaling via the Src-family kinases, Lck and Fyn, influences T-cell activation, differentiation, and tolerance. *Immunol. Rev.* 226, 9–22 CrossRef Medline

30. Boggan, T. J., and Eck, M. J. (2004) Structure and regulation of Src family kinases. *Oncogene* 23, 7918–7927 CrossRef Medline

31. McCabe, J. B., and Berthiaume, L. G. (1999) Functional roles for fatty acylated amino-terminal domains in subcellular localization. *Mol. Biol. Cell* 10, 3771–3786 CrossRef Medline

32. Tournaviti, S., Hennemann, S., Terjung, S., Kitzing, T. M., Stegmaier, C., Ritterfeld, J., Walther, P., Grosse, R., Nickel, W., and Fackler, O. T. (2007) SH4-domain-induced plasma membrane dynamization promotes bleb-associated cell motility. *J. Cell Sci.* 120, 3820–3829 CrossRef Medline

33. Chu, P. H., Tsygankov, D., Breginski, M. E., Dagliyan, O., Gomez, S. M., Elston, T. C., Karginov, A. V., and Hahn, K. M. (2014) Engineered kinase activation reveals unique morphodynamic phenotypes and associated trafficking for Src family isoforms. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12420–12425 CrossRef Medline

34. Sigal, C. T., Zhou, W., Buser, C. A., McLaughlin, S., and Resh, M. D. (1994) Amino-terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* 91, 12253–12257 CrossRef Medline

35. Wolenen, A., Okamura, H., Rosenblatt, Y., and Resh, M. D. (1997) Palmitoylation of p59fyn is reversible and sufficient for plasma membrane association. *Mol. Biol. Cell* 8, 1159–1173 CrossRef Medline

36. Alland, L., Peseckis, S. M., Atherton, R. E., Berthiaume, L., and Resh, M. D. (1994) Dual myristylation and palmitoylation of Src family mem-
Block of SH4 domain anterograde transport by HIV-1 Nef

Nef interferes with host cell motility by degradation of Cofilin. Cell Host Microbe 6, 174–186 CrossRef Medline

Imle, A., Abraham, L., Tsopoulidis, N., Hoffland, B., Saksela, K., and Fackler, O. T. (2015) Association with PAK2 enables functional interactions of lentiviral Nef proteins with the exocyst complex. mBio 6, e01309-15 Medline

Baur, A. S., Sass, G., Laffert, B., Willbold, D., Cheng-Mayer, C., and Peterlin, B. M. (1997) The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase. Immunity 6, 283–291 CrossRef Medline

Muratori, C., Cavallini, L. E., Krätzl, K., Tinari, A., De Milito, A., Fais, S., D’Alò, P., Federico, M., Vullo, V., Fominia, A., Mesri, E. A., Superti, F., and Baur, A. S. (2009) Massive secretion by T cells is caused by HIV-Nef infected cells and by Nef transfer to bystander cells. Cell Host Microbe 6, 218–230 CrossRef Medline

Lee, J. H., Wittki, S., Bräu, T., Dreyer, F. S., Krätzl, K., Dindorf, J., Johnston, I. C., Gross, S., Kremmer, E., Zeidler, R., Schützer-Schrehardt, U., Lichtenheld, M., Saksela, K., Harrer, T., Schuler, G., et al. (2013) HIV Nef, paxillin, and Pak1 regulate activation and secretion of TACE/ADAM10 proteases. Mol. Cell 49, 668–679 CrossRef Medline

Ostalecki, C., Wittki, S., Lee, J. H., Geist, M. M., Tibroni, N., Harrer, T., Schuler, G., Fackler, O. T., and Baur, A. S. (2016) HIV Nef- and Nef-dependent endocytosis of ADAM17 induces vesicular TNF secretion in chronic HIV infection. EBioMedicine 13, 294–304 CrossRef Medline

Trautz, B., Pierini, V., Wombacher, R., Stolp, B., Chase, A. J., Pizzato, M., and Fackler, O. T. (2016) The antagonism of HIV-1 Nef to SERINC1 particle infectivity restriction involves the counteraction of virion-associated pools of the restriction factor. J. Virol. 90, 10915–10927 CrossRef Medline

Cen, O., Gorska, M. M., Stafford, S. I., Sur, S., and Alam, R. (2003) Identification of UNC119 as a novel activator of SRC-type tyrosine kinases. J. Biol. Chem. 278, 8837–8845 CrossRef Medline

Lingwood, D., and Simons, K. (2007) Detergent resistance as a tool in membrane research. Nat. Protoc. 2, 2159–2165 CrossRef Medline

Schmidt, S., Fritz, J. V., Bitzegeio, I., Fackler, O. T., and Kepper, O. T. (2011) HIV-1 Vpu blocks recycling and biosynthetic transport of the intrinsic immunity factor CD317/tetherin to overcome the virus release restriction. mBio 2, e00306-11 Medline

Pujol, F. M., Laketa, V., Schmidt, F., Mukenhirn, M., Müller, B., Boulant, S., Grimm, D., Kepper, O. T., and Fackler, O. T. (2016) HIV-1 Vpu antagonizes CD317/Tetherin by adaptor protein-1-mediated exclusion from virus assembly sites. J. Virol. 90, 6709–6723 CrossRef Medline

Sandländers, E., Cans, C., Fincham, V. J., Brunton, V. G., Mellor, H., Prenberg, G. C., Norman, J. C., Superti-Furga, G., and Frame, M. C. (2004) RhoB and actin polymerization coordinate Src activation with endosomes-mediated delivery to the membrane. Dev. Cell 7, 855–869 CrossRef Medline

Reinecke, J., and Caplan, S. (2014) Endocytosis and the Src family of non-receptor tyrosine kinases. Biomat. Concepts 5, 143–155 Medline

Madrid, R., Janvier, K., Hitchin, D., Day, J., Coleman, S., Noviello, C., Bouchet, J., Benmerah, A., Guatelli, J., and Benichou, S. (2005) Nef-induced alteration of the early/recycling endosomal compartment correlates with enhancement of HIV-1 infectivity. J. Biol. Chem. 280, 5032–5044 CrossRef Medline

Mazzolini, J., Herit, F., Bouchet, J., Benmerah, A., Benichou, S., and Niebergang, F. (2010) Inhibition of phagocytosis in HIV-1-infected macrophages relies on Nef-dependent alteration of focal delivery of recycling compartments. Blood 115, 4226–4236 CrossRef Medline

Baldauf, H. M., Pan, X., Erikson, E., Schmidt, S., Daddawa, W., Burgraff, M., Schenkova, K., Ambiel, I., Wabnitz, G., Gramberg, T., Panitz, S., Florý, E., Landau, N. R., Sertel, S., Rutsch, F., et al. (2012) SAMHD1 restricts HIV-1 infection in resting CD4+(+) T cells. Nat. Med. 18, 1682–1687 CrossRef Medline

Newman, L. E., Zhou, C. J., Mudignon, S., Mattheyses, A. L., Paradis, E., Marobbio, C. M., and Kahn, R. A. (2014) The ARL2 GTPase is required for mitochondrial morphology, motility, and maintenance of ATP levels. PLoS ONE 9, e99270 CrossRef Medline
Block of SH4 domain anterograde transport by HIV-1 Nef

88. Rudolph, J. M., Eickel, N., Haller, C., Schindler, M., and Fackler, O. T. (2009) Inhibition of T-cell receptor-induced actin remodeling and relocalization of Lck are evolutionarily conserved activities of lentiviral Nef proteins. J. Virol. 83, 11528–11539 CrossRef Medline

89. Schmidt, S., Schenkova, K., Adam, T., Erikson, E., Lehmann-Koch, J., Sertel, S., Verhasselt, B., Fackler, O. T., Lasitschka, F., and Keppler, O. T. (2015) SAMHD1’s protein expression profile in humans. J. Leukocyte Biol. 98, 5–14 CrossRef Medline

90. Trotard, M., Tsopoulidis, N., Tibroni, N., Willemsen, J., Binder, M., Ruggieri, A., and Fackler, O. T. (2016) Sensing of HIV-1 infection in Tzm-bl cells with reconstituted expression of STING. J. Virol. 90, 2064–2076 Medline