Evidence of a Role for Soluble N-Ethylmaleimide-sensitive Factor Attachment Protein Receptor (SNARE) Machinery in HIV-1 Assembly and Release

Anjali Joshi†, Himanshu Garg‡§, Sherimay D. Ablan¶, and Eric O. Freed†

From the †Center of Excellence for Infectious Diseases, Department of Biomedical Sciences, and ‡Department of Pediatrics, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, Texas 79905 and the §Virus-Cell Interaction Section, HIV Drug Resistance Program, NCI, National Institutes of Health, Frederick, Maryland 21702-1201

Retrovirus assembly is a complex process that requires the orchestrated participation of viral components and host-cell factors. The concerted movement of different viral proteins to specific sites in the plasma membrane allows for virus particle assembly and ultimately budding and maturation of infectious virions. The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins constitute the minimal machinery that catalyzes the fusion of intracellular vesicles with the plasma membrane, thus regulating protein trafficking. Using siRNA and dominant negative approaches we demonstrate here that generalized disruption of the host SNARE machinery results in a significant reduction in human immunodeficiency virus type 1 (HIV-1) and equine infectious anemia virus particle production. Further analysis of the mechanism involved revealed a defect at the level of HIV-1 Gag localization to the plasma membrane. Our findings demonstrate for the first time a role of SNARE proteins in HIV-1 assembly and release, likely by affecting cellular trafficking pathways required for Gag transport and association with the plasma membrane.
tion during cytokinesis (39–44). The SNARE proteins constitute the minimal machinery for membrane fusion and are required for each step of the exocytic as well as endocytic trafficking pathways (44). Specific interactions between vesicle-associated (v) and target membrane-associated (t) SNAREs lead to formation of a trans-SNARE complex that causes fusion of apposing membranes, thereby mediating cargo delivery. SNARE complex function, assembly, and disassembly are regulated by SNARE-associated factors. Once assembled, the SNARE complexes are recycled by N-ethylmaleimide-sensitive factor (NSF), an AAA family ATPase chaperone, and its adapter protein α-soluble NSF attachment protein (SNAP) which binds directly to the SNARE complex (45). A dominant negative (DN) disruption of SNARE activity occurs on mutation of the ATP binding site (E329Q) of NSF that results in failure of NSF to disassemble the SNAP-SNARE complex due to a significant decrease in NSF ATPase activity (46–48).

Although the role of SNAREs in specific cellular functions like vesicle-mediated transport, exocytosis, and endocytosis is well characterized, the function of this group of proteins in retrovirus assembly and budding remains undetermined. Hence, in the current study, we used specific siRNAs against SNAP-23 and NSF as well as the DN NSF mutant E329Q to investigate the role of these proteins in retrovirus particle production. We find that SNAP-23 or NSF depletion and NSF-DN expression lead to defects in HIV-1 particle production. This defect correlates with a deficiency in Gag-membrane localization, suggesting a role for SNARE proteins in Gag accumulation at the PM. Moreover, expression of NSF-DN also leads to dissociation of MVB markers CD63 and CD81 from membranes. These data support a role for the SNARE proteins in retrovirus assembly and release.

**MATERIALS AND METHODS**

**Cell Culture and Transfections**—HeLa cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and 2 mM glutamine. The CD4+/CXCR4+/CCR5+ HeLa cell derivative TZM-bl (49), obtained from J. Kappes through the AIDS Research and Reference Reagent Program, and 293T cells were cultured in DMEM supplemented with 10% FBS. All transfections were performed using Lipofectamine 2000™ reagent (Invitrogen) according to the manufacturer’s instructions.

**Plasmids, Antibodies, Cell Culture Reagents, and siRNAs**—The full-length HIV-1 proviral clone pNL4-3 (50) and the pNL4-3/K29E/K31E derivative (51, 52) have been described. The vpu-deficient (delVpu) molecular clone pNL4-3/U35 (53) was kindly provided by K. Strebel (NIAID/NIH). An HIV-1 derived bearing at its N terminus 10 amino acids from the membrane-targeting sequence of Fyn (Fyn10fullMA) (54) and its derivative lacking the MA domain (Fyn10deltaMA) (18) have been described. Vesicular stomatitis virus G glycoprotein-pseudotyped virus stocks were prepared by transfecting 293T cells with pCMVNLGagPolIRE, pHCMV-G, and pNL4-3-derived molecular clones (51, 55). The EIAV Gag expression vector pPRE/Gag has been reported previously (56, 57). The MLV Gag expression vector was obtained through the AIDS reference reagent program (58). WT NSF was cloned from HeLa cDNA using specific primer pairs bearing an N-terminal HA tag using the pcDNA™ 3.1 Directional TOPO Expression kit (Invitrogen) and sequenced. Primer pairs and sequences will be made available on request. The ATPase-defective DN NSF mutant (E329Q) was constructed by QuickChange site-directed mutagenesis (Stratagene) following the manufacturer’s instructions.

NSF (Qiagen, catalog number SI03019303) and SNAP-23 (Qiagen, custom) siRNAs were purchased. NSF and SNAP-23 knock-down efficiencies were determined by transfecting HA-tagged NSF and SNAP-23 expression vectors along with the corresponding siRNAs and determining protein levels by anti-HA Western blotting. Western blots were quantified using Quantity One software. Anti-EIAV serum was kindly provided by R. Montelaro (University of Pittsburgh) and HIV immunoglobulin (HIV-Ig) and HIV-1 gp120 monoclonal antibody (B12) were provided by the NIH AIDS Research and Reference Reagent Program. Protein A beads were from Invitrogen. The p24 (CA) monoclonal was from Abcam (39/5.4A) and mouse anti-CD63, anti-CD81, and rabbit anti-HA antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The CD4-FITC antibody was from Beckman Coulter and the CCR5-PE antibody was from BD Biosciences. Cytochalasin D (CytoD) and brefeldin A (BFA) were purchased from Sigma. Carboxyfluorescein succinimidyl ester (CFSE) and membrane dye Dil were from Invitrogen. With each cell division the CFSE dye is diluted 2-fold as it is distributed equally into each daughter cell and hence can be used to monitor cell divisions. Cell cycle analysis was conducted by CFSE dilution experiments and cells were acquired using the FACS CANTO-II flow cytometer. Data were analyzed using FlowJo software with at least 20,000 events acquired for each sample.

**Immunofluorescence**—Immunostaining of cells was performed as described (59), with minor modifications. Cells seeded onto Nunc Lab-Tek II chamber slides were rinsed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 20 min. Following fixation, cells were thoroughly rinsed with PBS, permeabilized using 0.1% Triton X-100/PBS for 2 min, and incubated for 10 min with 0.1 M glycine/PBS to quench the remaining aldehyde residues. Cells were then blocked with 3% BSA/ PBS for 30 min, followed by incubation for 1 h with primary antibody appropriately diluted in 3% BSA/PBS. After three washes in PBS, cells were incubated for 30 min with secondary antibody diluted in 3% BSA/PBS. Cells were then washed and mounted using Prolong antifade reagent (Invitrogen). For detection of HIV-1 Env glycoprotein expression, cells were stained using the anti-gp120 antibody before fixation as described (60). Images were acquired using the NikonTi microscope.

**Metabolic Labeling, Immunoprecipitation, and Cell Fractionation**—The protocol for radiolabeling and immunoprecipitation of cell and virus lysates has been described in detail previously (61). Briefly, transfected cells were starved for 30 min in RPMI 1640 medium lacking Met and Cys. Thereafter, cells were incubated for 2–3 h in RPMI 1640 medium supplemented with FBS and [35S]Met/Cys. Culture supernatants were filtered and subjected to ultracentrifugation at 100,000 × g for 45 min; cell and virus lysates were immunoprecipitated with HIV-Ig and...
resolved by SDS-PAGE followed by PhosphorImager analysis. Virus release efficiency = virion p24/(cell-associated Pr55^{CA} + cell-associated p24 + virion-associated p24) × 100. Membrane and cytoplasmic fractions were isolated using the Subcellular Protein Fractionation kit (Pierce) strictly following the manufacturer’s protocol. Isolated fractions were lysed with 2 × radioimmunoprecipitation buffer (280 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, 2% NP-40, 1% sodium deoxycholate, 0.1% SDS, 20 mM iodoacetoamide) before loading on gels for Western blotting.

RESULTS

Generalized Disruption of the SNARE Machinery Inhibits Retrovirus Particle Production—The involvement of both ESCRT and SNARE machinery in cytokinesis prompted us to investigate a potential role for SNARE proteins in retrovirus budding. To determine whether SNARE machinery functions in the HIV-1 assembly/release pathway, we used an siRNA-based approach to deplete cells of NSF or SNAP-23, both of which are critical components of the SNARE machinery. NSF is an ATPase required for disassembly of the cis-SNARE complex and subsequent recycling of v- and t-SNARE components (40, 43, 62). SNAP-23 is a t-SNARE that interacts with a number of v-SNAREs, including VAMP-2, -3, -7, and -8 to form functional cis-SNARE complexes (40, 63, 64). HeLa cells were transfected with siRNAs specific for NSF or SNAP-23, and the effects on HIV-1 particle production were determined by metabolic radiolabeling and immunoprecipitation analysis. Knock-down efficiencies were in the range of 65–80% (Fig. 1A, bottom), and transfection with the above siRNAs was minimally toxic as determined by comparable Gag expression levels in cell lysates in Fig. 1A. Interestingly, we observed that depletion of either NSF or SNAP-23 led to a marked reduction in HIV-1 particle production (Fig. 1A). We extended these findings by disrupting the function of SNARE proteins with a DN NSF mutant (NSF-DN), E329Q, defective in ATP hydrolysis (46, 47). As shown in Fig. 1B, expression of NSF-DN but not its WT counterpart (NSF-WT) inhibited HIV-1 particle production. Moreover, NSF-DN expression led to a Gag-processing defect, characterized by a significant (2–3-fold) increase in the ratio of Pr55^{CA} (Pr55) to p24 (CA) (Fig. 1B).

Defective Gag processing is often associated with impaired Gag-membrane binding (e.g. 22, 59, 65, 66). We next investigated whether the inhibition mediated by NSF disruption was HIV-1-specific or whether it extended to other retroviruses. To this end, we tested the effect of NSF overexpression on HIV-1 Gag processing, as measured by the ratio of Pr55^{CA} (Pr55) to p24 (CA) is also shown. Data represent mean ± S.D. (error bars), n = 5 (A and B) or 3 (C and D).

FIGURE 1. Generalized disruption of the SNARE machinery inhibits retrovirus release. A, top, HeLa cells were transfected with control, NSF, or SNAP-23 siRNA. 24 h later, cells were infected with HIV-1 particles pseudotyped with vesicular stomatitis virus G. 24 h after infection, cells were labeled with [35S]Met/Cys for 3 h. Virus was pelleted by ultracentrifugation, and cell virions were immunoprecipitated with HIV-1 Ig and subjected to SDS-PAGE and fluorography. Positions of the Gag precursor protein Pr55^{CA} (Pr55) and mature CA (p24) are indicated. Relative virus release efficiency (virus release) was calculated as described under “Materials and Methods.” Bottom, efficiency of NSF and SNAP-23 siRNA-mediated depletion is shown. HeLa cells were transfected with HA-tagged NSF or SNAP-23 along with the control, NSF-, or SNAP-23-specific siRNA. Cell lysates were analyzed by anti-HA Western blotting (WB). B–D, WT HIV-1 (B) or EIAV (C), or MLV expression vectors (D) were cotransfected with control vector or vectors expressing HA-tagged NSF-WT or NSF-DN at a DNA ratio of 4:1. Virus release was determined as described above; anti-EIAV horse antiserum was used for the EIAV Gag IP, and goat anti-MLV serum was used for MLV Gag IP. In B, the effect of NSF overexpression on HIV-1 Gag processing, as measured by the ratio of Pr55^{CA} (Pr55) to p24 (CA) is also shown.
cessing and release. To determine which domain of HIV-1 Gag is sensitive to NSF-DN-mediated inhibition, we performed virus release assays with an HIV-1 proviral clone lacking either the MA (Fyn10DelMA), NC (Fyn10DelNC), or both MA and NC domains (Fyn10DelMApDelNC) (Fig. 2A) (18). The absence of MA removes the myristylation sequence that is indispensable for membrane binding, and hence we used constructs in which the 10 N-terminal amino acids from the PM-associated Fyn protein (light green), which bears one myristylation (M) and two palmitylation (Palm) signals. In each case, virus release efficiency was normalized to the levels obtained in the presence of NSF-WT expression and was set at a relative value of 100%. C, HeLa cells cotransfected with pNL4-3 clones expressing WT Gag or the Fyn10-tagged derivatives and HA-tagged NSF-WT or NSF-DN at a DNA ratio of 1.6:1. Cells were fixed 24 h after transfection, stained with anti-p24 (green) and anti-HA antibodies (red), and analyzed by fluorescence microscopy. Nuclei are shown in blue (DAPI).
Gag derivative with a high membrane binding ability. As a control, we also used a Gag chimera (Fyn10fullMA) which retains the entire MA sequence following the Fyn-derived peptide (Fig. 2A). Interestingly, whereas NSF-DN expression resulted in reduced production of WT, Fyn10fullMA, and Fyn10DelMA particles, it had no effect on release of Fyn10DelNC or Fyn10DelMADelNC Gag (Fig. 2B). These results indicate that removal of the NC domain reverses the effect of NSF-DN on particle production. We note that the overall release efficiency of the NC-deleted Gag mutants is much lower than that of the constructs encoding intact NC.

We next analyzed the Gag staining pattern in cells expressing the above described Fyn10 chimeric Gag derivatives using an anti-HIV-1 p24 antibody (Fig. 2C; NSF, red; Gag, green). NSF-DN but not NSF-WT altered the Gag localization pattern from punctate PM to diffuse cytosolic. This alteration in Gag localization was clearly evident for WT, Fyn10fullMA, and Fyn10DelMA, all of which are membrane-localized, whether it be to the PM (WT and Fyn10fullMA) or to internal MVB-like compartments (Fyn10DelMA) (Fig. 2C). However, the predominantly diffuse localization for Fyn10DelNC or Fyn10DelMADelNC Gag was not significantly altered by NSF-WT or DN expression (Fig. 2C). Thus, the alteration in WT, Fyn10fullMA, and Fyn10DelMA Gag localization from PM to cytosolic correlated with reduced virus release. These results suggest that NSF-DN inhibits virus particle production by disrupting Gag-membrane localization.

To expand on these results, we conducted virus release assays with various HIV-1 mutants that differ from the WT in their Gag localization or particle release patterns: the MVB-targeted MA mutant K29E/K31E (19, 23, 52), the Vpu-deficient mutant K29E/K31E DelVpu (53), and the PTAP− late domain mutant (8). These mutants are all deficient in virus production in HeLa cells for different reasons. The K29E/K31E mutant accumulates Gag in MVBs (19, 23); the DelVpu mutation results in the tethering of released virions to the cell surface and in endosomes (67); and elimination of the PTAP late domain blocks the pinching off of particles from the PM (8, 9). Interestingly, we observed that NSF-DN inhibited virus particle production for all of these HIV-1 mutants (Fig. 3A). To understand further the mechanism behind this phenomenon we determined the Gag localization pattern of the mutants by CA (p24) staining when expressed in the presence of NSF-WT or NSF-DN. Interestingly, again, fluorescence microscopy analysis and quantitation of data suggested that NSF-DN shifted the Gag localization pattern for all mutants from punctate to diffuse cytosolic (Fig. 3B). For p24 staining we also included the myristoylation-deficient 1GA mutant which is severely defective in virus production due to lack of Gag membrane binding. We found that the Gag localization pattern for the 1GA mutant was not altered in the presence of NSF-DN. Together, these data suggest that NSF-DN inhibits virus release by disrupting Gag-membrane localization.

Our virus release results indicate that NSF-DN inhibits the production of both WT HIV-1 and the K29E/K31E MA mutant particles. This MA mutant exhibits a predominantly intracellular Gag staining pattern and colocalizes significantly with the tetraspanins CD63 and CD81 (51, 68, 69), which are markers for late endosomes and MVBs (70–72). We thus looked at the effect of NSF-DN expression on CD63 and CD81 localization.

Interestingly, we observed that expression of NSF-DN but not NSF-WT shifted the localization of CD63 (Fig. 4A) and CD81 (Fig. 4B) from a punctate, MVB-like pattern to a predominantly cytosolic pattern. We confirmed these results by isolating membrane and cytosolic fractions from NSF-WT- or NSF-DN-expressing cells and analyzing for CD63 expression. Consistent with the microscopy data we found that in mock-transfected or NSF-WT vector-transfected cells essentially all the CD63 fractionated with membrane. In contrast, in cells expressing NSF-DN approximately half of the detected CD63 localized to the cytosolic fraction (Fig. 4C). The changes in localization of these MVB markers are strikingly similar to the alteration in Gag localization by NSF-DN. These results suggest that NSF-DN disrupts the association of these tetraspanins with the MVB membrane.

NSF-DN Disrupts Env Glycoprotein Processing and Virion Infectivity—In the course of performing the virus release assays presented above, it became evident that expression of NSF-DN not only inhibited virus release and Gag processing (Fig. 1) but also led to defects in envelope (Env) glycoprotein processing. The HIV-1 Env glycoprotein precursor, gp160, traffics through the secretory pathway and is processed into gp120 and gp41 subunits in the Golgi apparatus by a cellular furin-like protease (for review, see Footnote 4). Quantitation of the gp160/120 ratio in cells not overexpressing NSF or overexpressing NSF-WT or -DN demonstrated a severe gp160-processing defect induced by NSF-DN (Fig. 5A). This is consistent with a role for the SNARE proteins in Golgi trafficking (74). To determine whether this gp160-processing defect impaired virion infectivity, we performed single-round infection assays in the TZM-bl indicator cell line (49) using virus produced from HeLa cells expressing either NSF-WT or NSF-DN. As shown in Fig. 5B, virions derived from HeLa cells expressing NSF-DN displayed significantly reduced specific infectivity relative to virions produced from cells expressing NSF-WT. We further looked at Env localization in NSF-DN-expressing cells. Interestingly, although there were a large number of cells showing surface Env staining in control and NSF-WT-expressing cells, very little surface Env expression was detected in cells transfected with the NSF-DN vector. Instead, Env in these cells was localized to an intracellular site, similar to that seen with BFA-treated cells (Fig. 5C). Thus, NSF-DN expression not only causes defects in virion release but also induces a defect in surface Env localization, gp160 processing, and consequently virion infectivity.

Virus Assembly Defects Induced by NSF-DN Are Distinct from the Effects of the Golgi Inhibitor BFA or the Actin Polymerization Inhibitor CytoD—It has been shown previously that NSF-DN causes defects in vesicular transport, particularly within the Golgi, due to its failure to hydrolyze ATP (48, 74). Moreover, NSF-DN has also been suggested to induce changes in cytoskeletal organization (62). To examine whether alterations in membrane trafficking or the cytoskeleton could account for the defects in particle production induced by NSF-DN, we tested

---

Footnote 4: M. A. Checley, B. G. Lutlge, and E. O. Freed, submitted for publication.
the effects on particle production of two agents: the fungal toxin BFA, which disrupts ER-to-Golgi trafficking (75), and CytoD, which blocks actin polymerization (76). Neither BFA (consistent with Ref. 22) nor CytoD had an effect on HIV-1 (Fig. 6A) or EIAV release (data not shown). As expected (77), BFA inhibited gp160 processing (Fig. 6A), and CytoD induced changes in cell morphology evident by cell rounding, similar to, but more severe than those induced by NSF-DN (Fig. 6C). Furthermore, CytoD also caused defects in cell division, as indicated by a lack of CFSE dilution, whereas neither NSF-DN nor NSF-WT affected cell division in this assay (Fig. 6B). We also looked at the cell surface expression of CD4 and CCR5 in the presence of NSF-DN, both of which traffic through the Golgi pathway (78). Our data indicate that expression of NSF-DN reduces cell surface expression of both CD4 (Fig. 6D) and CCR5 (not shown). Taken together, these findings suggest that although SNARE disruption induced by NSF-DN may lead to global impairment in vesicular trafficking, defects in Golgi trafficking and the actin cytoskeleton induced by NSF-DN are not directly responsible for the inhibition of particle production.

**DISCUSSION**

HIV-1 assembly and release involve a complex and concerted interplay between host cell factors and virion components. Although multiple domains in HIV-1 Gag play specific and well defined roles in particle production, the participation of host SNAREs in HIV Assembly

**FIGURE 3. NSF-DN inhibits the production of K29E/K31E MA-mutant and late domain-defective HIV-1.** A, HeLa cells were cotransfected with WT pNL4-3 or derivatives expressing the K29E/K31E MA mutant or PTAPlate domain mutant and vectors expressing NSF-WT (lane 1) or NSF-DN (lane 2) at a DNA ratio of 4:1. Cells were metabolically radiolabeled, and virus and cell lysates were prepared and analyzed as described under "Materials and Methods" and in the Fig. 1 legend. Data represent means ± S.D. (error bars), $n = 3$. B, HeLa cells were cotransfected with the indicated pNL4-3 derivatives and vectors expressing HA-tagged WT or DN NSF at a DNA ratio of 1.6:1. Cells were fixed 24 h after transfection, stained with anti-p24 and anti-HA antibodies, and analyzed by fluorescence microscopy. Graph represents quantitation of diffuse or punctate Gag staining pattern in NSF-WT- or NSF-DN-expressing cells, $n = 30$. Cells with more than 75% diffuse or punctate Gag staining were scored as diffuse versus punctate, respectively.
factors in HIV-1 assembly and release continues to be an active area of investigation. In this respect, we examined the role of SNARE machinery in retrovirus assembly and release. The SNARE proteins are important for fusion of intracellular transport vesicles and hence are key components in vesicle-mediated transport events. Our findings suggest a role, either direct or indirect, for SNARE proteins in the HIV-1 assembly pathway by affecting Gag trafficking and localization to the PM. To the best of our knowledge, this is the first study demonstrating the importance of the SNARE machinery in retrovirus assembly and budding.

The itinerary that retroviral Gag proteins follow en route to the PM is poorly characterized, and it remains unclear whether, and to what extent, vesicle trafficking pathways contribute to Gag transport (for review, see Ref. 36). It is widely accepted that in most cell types, HIV-1 assembly occurs predominantly at the

FIGURE 4. NSF-DN induces the cytosolic localization of CD63 and CD81. A and B, HeLa cells were transfected with vectors expressing HA-tagged NSF-WT or NSF-DN. 24 h after transfection, cells were fixed, stained with anti-HA antibody along with anti-CD63 (A) or anti-CD81 antibody (B) and analyzed by fluorescence microscopy. C, HeLa cells were transfected with control pcDNA3.1, NSF-WT, or NSF-DN expression vectors. Cells were harvested approximately 24 h after transfection, and membrane and cytosolic fractions were isolated using the Subcellular Protein Fractionation kit (Pierce) and analyzed for CD63 expression using Western blotting. M, membrane fraction; C, cytosolic fraction; L, protein ladder. One representative of two independent experiments is shown.

FIGURE 5. Expression of NSF-DN leads to defects in HIV-1 Env glycoprotein processing and virion infectivity. A, HeLa cells were cotransfected with WT pNL4-3 and vectors expressing NSF-WT or NSF-DN as described previously (DNA ratio 4:1). 24 h after transfection cells were radiolabeled and processed as described in the Fig. 1 legend. Data represent mean ± S.D. (error bars), n = 3. This panel represents a portion of Fig. 1B. B, HeLa cells were transfected as described in A. Virus supernatants were collected 24 h after transfection, normalized for reverse transcriptase activity, and used to infect the TZM-bl indicator cell line. Luciferase activity was measured 24 h after infection. Data are mean ± S.D. of triplicate observations from three independent experiments. C, HeLa cells were transfected with the WT HIV-1 clone along with control or HA-tagged NSF-WT or NSF-DN expression constructs. 24 h later the indicated wells were treated with 2 μg/ml BFA for 1 h. Cells were then incubated on ice for 5 min followed by staining with HIV-1 anti-gp120 antibody and anti-human Alexa Fluor 594 antibody on ice. Cells were then fixed, stained with anti-HA antibody, and analyzed by fluorescence microscopy. Results are representative of 20–35 cells scored for each treatment.
PM (1, 51, 79–82). However, several groups have proposed that late endosomal compartments serve as major or primary sites for HIV-1 assembly (71, 83–86, 88), and a number of cellular proteins and protein complexes implicated in protein sorting to and from the endosomal pathway have been implicated in Gag trafficking and assembly (22, 24–26). Moreover, Molle et al. recently reported that HIV-1 Gag and viral genomic RNAs are transported on vesicles that are positive for the v-SNARE TiVamp (89). In our study, we found that disruption of SNARE function using NSF-DN affected Gag localization and particle production for both WT Gag, which assembles predominantly at the PM, and K29E/K31E mutant Gag, which is localized primarily to late endosomes/MVBs. Because SNARE proteins are involved in vesicular trafficking and fusion events it is conceivable that both WT and K29E/K31E mutant Gag traffic via the endosomal pathway and hence are both affected by disruption of SNARE
function. However, it is also possible that SNARE disruption acts on Gag indirectly by altering the localization or activity of cellular factors that play more proximal roles in Gag trafficking. In this context, Jouvenet et al. (81) demonstrated that agents that block vesicle motility did not affect HIV-1 particle production. SNARE proteins are reported to interact with PI(4,5)P₂ (90), which also plays an important role in recruiting HIV-1 Gag to the PM (18–20) and also localize to lipid rafts (91) where HIV-1 Gag assembles (for review, see Ref. 87). NSF-DN expression could thus disrupt some aspect of Gag targeting to PI(4,5)P₂ and/or lipid rafts. Based on our findings in this study we propose a model showing the potential mechanism of NSF-DN-mediated inhibition of HIV assembly (Fig. 7).

Our data demonstrate that generalized disruption of SNARE machinery inhibits retroviral particle production. The modest effects of NSF-DN expression on MLV versus HIV and EIAV release could be due to use of different mechanisms and host factors in the trafficking and assembly of these Gag proteins. For instance, HIV-1, MLV, and EIAV use different proteins to connect to the cellular ESCRT pathway during virus budding and release (4, 16, 17), and MLV is less strongly inhibited by GGA overexpression relative to HIV-1 and EIAV (22, 23). Disruption of NSF via its DN counterpart induced an accumulation of HIV-1 Gag precursor, inhibited Env glycoprotein processing, and impaired virus assembly and release. Interestingly, we observed that Gag derivatives lacking the NC domain, which plays a central role in promoting Gag-Gag interactions required for higher order Gag multimerization during assembly, were not disrupted by NSF-DN. These data suggest that the NC domain itself, or higher order Gag oligomerization directed by the NC domain, is the target of NSF-DN disruption. The NC domain of HIV-1 Gag was recently shown to be responsible for Gag localization to the uropod and virological synapse, a function that could be bypassed by replacing NC with a foreign leucine zipper dimerization motif (60). Although the SNARE machinery regulates numerous cellular functions in our study, we were able to demonstrate that defects in cellular morphology and Golgi trafficking induced by NSF-DN were not responsible for the disruption of HIV assembly as recapitulation of these defects in cells by chemical means failed to affect retrovi-

---

**FIGURE 7. Hypothetical model for the action of NSF-DN on retroviral particle production.** HIV-1 Gag (brown circle) is shown to traffic to the PM via a direct route or via late endosomes or transport vesicles. Env glycoproteins (blue lollipops) traffic to the PM via the ER-Golgi secretory pathway. Gag is targeted to cholesterol-enriched membrane raft microdomains (blue bar) and PI(4,5)P₂ (yellow star) at the PM. NSF-DN is proposed to inhibit the function of t- and v-SNAREs and intra-Golgi membrane traffic.
SNAREs in HIV Assembly

ral budding. This suggests that the role played by the SNARE machinery in HIV assembly is likely more specific than generalized disruption of cellular functions.

In conclusion, our findings suggest an important role for the SNARE proteins in HIV assembly and release. Further studies will define more precisely the mechanism by which these proteins stimulate virus particle production.

Acknowledgments—We thank members of the Freed laboratory for helpful discussion and critical review of the manuscript. The HIV-1g and TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. We thank A. Ono for the Fyn10 Gag constructs and R. Montelaro for providing horse anti-EIAV antiserum.

REFERENCES

1. Swanstrom, R., and Wills, J. W. (1997) in Retroviruses (Coffin, J. M., Hughes, S. H., and Varumus, H. E., eds) pp. 263–334, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
2. Vogt, V. M. (1997) in Retroviruses (Coffin, J. M., Hughes, S. H., and Varumus, H. E., eds) pp. 27–70, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
3. Adamson, C. S., and Freed, E. O. (2007) Adv. Pharmacol. 55, 347–387.
4. Bieniasz, P. D. (2006) Virology 344, 55–63.
5. Freed, E. O. (1998) Virology 251, 1–15.
6. Goff, S. P. (2001) Annu. Rev. Cell Biol. 15, 227–238.
7. Goff, S. P. (2001) in The Retroviruses and Their Replication (Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B., and Straus, S. E., eds), 4th ed., Lippincott Williams & Wilkins, Philadelphia, PA.
8. Joshi, A., and Freed, E. (2007) Future Med. 1, 427–438.
9. Huang, M., Orenstein, J. M., Martin, M. A., and Freed, E. O. (1995) J. Virol. 69, 6810–6818.
10. Demirov, D. G., Orenstein, J. M., and Freed, E. O. (2002) J. Virol. 76, 105–117.
11. Martin-Serrano, J., Zang, T., and Bieniasz, P. D. (2003) Vol. 286, 91–101.
12. VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J., and Carter, C. A. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 7774–7779.
13. Martin-Serrano, J., and Bieniasz, P. D. (2003) J. Virol. 77, 12373–12377.
14. Strack, B., Calistri, A., Craig, S., Popova, E., and Göttlinger, H. G. (2003) Cell 114, 689–699.
15. Alroy, I., Tuvia, S., Greener, T., Gordon, D., Barr, H. M., Taglicht, D., Mandil-Levin, R., Ben-Avraham, D., Konforty, D., Nir, A., Levius, O., Bicoviski, V., Dori, M., Cohen, S., Yaar, L., Erez, 0., Prophet-Meiran, O., Koskas, M., Caspi-Bachar, E., Alchanati, I., Sela-Brown, A., Moskowitz, H., Tessmer, U., Schubert, U., and Reiss, Y. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 1478–1483.
16. Morita, E., and Sundquist, W. I. (2004) J. Virol. 82, 9937–9950.
17. Tang, Y., Winkler, U., Freed, E. O., Torrey, T. A., Kim, W., Li, H., Goff, S. P., and Morse, H. C., 3rd (1999) J. Virol. 73, 10508–10513.
18. Zimmerman, C., Klein, K. C., Kiser, P. K., Singh, A. R., Firestein, B. L., Riba, S. C., and Lingappa, J. R. (2002) Nature 415, 88–92.
19. Chatel-Chaix, L., Abrahamyan, L., Frêchita, C., Mouland, A. J., and Des-Groseillers, L. (2007) J. Virol. 81, 6216–6230.
20. Harrist, A. V., Ryzhova, E. V., Harvey, T., and Gonzalez-Scarano, F. (2009) PLoS One 4, e5020.
21. Ryzhova, E. V., Vois, R. M., Albright, A. V., Harrist, A. V., Harvey, T., and Gonzalez-Scarano, F. (2006) J. Virol. 80, 2694–2704.
22. Ehrlich, L. S., Medina, G. N., Khan, M. B., Powell, M. D., Mikhail, K., and Carter, C. A. (2010) J. Virol. 84, 6438–6451.
23. Deleted in proof.
24. Carlton, J. G., and Martin-Serrano, J. (2007) Science 316, 1908–1912.
25. Erlich, J. S., Medina, G. N., Khan, M. B., Powell, M. D., Mikhail, K., and Carter, C. A. (2010) J. Virol. 84, 6438–6451.
26. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) J. Virol. 72, 14889–14894.
27. Sumida, M., Hong, R. M., and Tagaya, M. (1994) J. Biol. Chem. 269, 20636–20641.
28. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Martin, M. A. (1986) J. Virol. 59, 2855–2866.
29. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) J. Virol. 72, 2855–2864.
30. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. A. (1986) J. Virol. 59, 284–291.
31. Ono, A., and Freed, E. O. (2004) J. Virol. 78, 1552–1563.
32. Ono, A., Orenstein, J. M., and Freed, E. O. (2000) J. Virol. 74, 2855–2866.
33. Strebel, K., Klimkait, T., Maldarelli, F., and Martin, M. A. (1989) J. Virol. 63, 3784–3791.
34. Ono, A., Waheed, A. A., and Freed, E. O. (2007) J. Virol. 81, 7435–7437.
35. Yee, J. K., Friedmann, T., and Burns, J. C. (1994) Methods Cell Biol. 43, 99–112.
36. Patnaik, A., Chau, V., Li, S., Montelaro, R. C., and Wills, J. W. (2002) J. Virol. 76, 2641–2647.
37. Heuß-Xilagia, M., Ablan, S., Demirov, D. G., Chen, C., Montelaro, R. C., and Freed, E. O. (2004) J. Virol. 78, 724–732.
38. Landau, N. R., and Littman, D. R. (1992) J. Virol. 66, 5110–5113.
39. Joshi, A., Nagashima, K., and Freed, E. O. (2006) J. Virol. 80, 7939–7951.
40. Llewellyn, G. N., Hogue, I. B., Grover, J. R., and Ono, A. (2010) PLoS Pathog 6, e1001167.
61. Freed, E. O., Orenstein, J. M., Buckler-White, A. J., and Martin, M. A. (1994) J. Virol. 68, 5311–5320
62. Zhao, C., Slevin, J. T., and Whiteheart, S. W. (2007) FEBS Lett. 581, 2140–2149
63. Kawanishi, M., Tamori, Y., Okazawa, H., Araki, S., Shinoda, H., and Kasuga, M. (2000) J. Biol. Chem. 275, 8240–8247
64. Okayama, M., Arakawa, T., Mizoguchi, I., Tajima, Y., and Takuma, T. (2007) FEBS Lett. 581, 4583–4588
65. Chang, Y. F., Wang, S. M., Huang, K. J., and Wang, C. T. (2007) J. Mol. Biol. 370, 585–597
66. Ono, A., and Freed, E. O. (1999) J. Virol. 73, 4136–4144
67. Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A., and Orenstein, J. M. (1990) J. Virol. 64, 621–629
68. Goussev, K., Ablan, S. D., Coren, L. V., Ono, A., Soheilian, F., Nagashima, K., Ott, D. E., and Freed, E. O. (2008) PLoS Pathog. 4, e1000015
69. Joshi, A., Ablan, S. D., Soheilian, F., Nagashima, K., and Freed, E. O. (2009) J. Virol. 83, 5375–5387
70. Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O., and Geuze, H. (1998) J. Biol. Chem. 273, 20121–20127
71. Pelchen-Matthews, A., Kramer, B., and Marsh, M. (2003) J. Cell Biol. 162, 443–455
72. Rappos, G., Moore, M., Innes, D., Leijendekker, R., Leigh-Brown, A., Benauch, P., and Geuze, H. (2002) Traffic 3, 718–729
73. Deleted in proof
74. Malhotra, V., Orci, L., Glick, B. S., Block, M. R., and Rothman, J. E. (1988) Cell 54, 221–227
75. Miller, S. G., Carnell, L., and Moore, H. H. (1992) J. Cell Biol. 118, 267–283
76. Dubinsky, W. P., Mayorga-Wark, O., and Schultz, S. G. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 9421–9426
77. Earl, P. L., Moss, B., and Doms, R. W. (1991) J. Virol. 65, 2047–2055
78. Piquet, V., Schwartz, O., Le Gall, S., and Trono, D. (1999) Immunol. Rev. 168, 51–63
79. Finzi, A., Orthwein, A., Mercier, J., and Cohen, E. A. (2007) J. Virol. 81, 7476–7490
80. Gelderblom, H. R. (1991) Aids 5, 617–637
81. Jouvenet, N., Neil, S. J., Bess, C., Johnson, M. C., Virgen, C. A., Simon, S. M., and Bieniasz, P. D. (2006) PLoS Biol. 4, e435
82. Rudner, L., Nydegger, S., Coren, L. V., Nagashima, K., Thali, M., and Ott, D. E. (2005) J. Virol. 79, 4055–4065
83. Gould, S. I., Booth, A. M., and Hildreth, J. E. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 10592–10597
84. Grigorov, B., Arcanger, F., Roingeard, P., Darlix, J. L., and Muriaux, D. (2006) J. Mol. Biol. 359, 848–862
85. Nguyen, D. G., Booth, A., Gould, S. J., and Hildreth, J. E. (2003) J. Biol. Chem. 278, 52347–52354
86. Nydegger, S., Foti, M., Derdowski, A., Spearman, P., and Thali, M. (2003) Traffic 4, 902–910
87. Waheed, A. A., and Freed, E. O. (2010) Viruses 2, 1146–1180
88. Sherer, N. M., Lehmann, M., Jimenez-Soto, L. F., Ingmundson, A., Horner, S. M., Cicchetti, G., Allen, P. G., Pypaert, M., Cunningham, J. M., and Mothes, W. (2003) Traffic 4, 785–801
89. Molle, D., Segura-Morales, C., Camus, G., Beriloz-Torrent, C., Kjems, L., Basuyuk, E., and Bertrand, E. (2009) J. Biol. Chem. 284, 19727–19743
90. James, D. J., Khodthong, C., Kowalchyk, J. A., and Martin, T. F. (2008) J. Cell Biol. 182, 355–366
91. Lang, T. (2007) J. Physiol. 585, 693–698