Filamin A Protein Interacts with Human Immunodeficiency Virus Type 1 Gag Protein and Contributes to Productive Particle Assembly

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HIV-1 Gag precursor directs virus particle assembly and release. In a search for Gag-interacting proteins that are involved in late stages of the HIV-1 replication cycle, we performed yeast two-hybrid screening against a human cDNA library and identified the non-muscle actin filament cross-linking protein filamin A as a novel Gag binding partner. The 280-kDa filamin A regulates cortical actin network dynamics and participates in the anchoring of membrane proteins to the actin cytoskeleton. Recent studies have shown that filamin A facilitates HIV-1 cell-to-cell transmission by binding to HIV receptors and coreceptors and regulating their clustering on the target cell surface. Here we report a novel role for filamin A in HIV-1 Gag intracellular trafficking. We demonstrate that filamin A interacts with the capsid domain of HIV-1 Gag and that this interaction is involved in particle release in a productive manner. Disruption of this interaction eliminated Gag localization at the plasma membrane and induced Gag accumulation within internal compartments. Moreover, blocking clathrin-dependent endocytic pathways did not relieve the restriction to particle release induced by filamin A depletion. These results suggest that filamin A is involved in the distinct step of the Gag trafficking pathway. The discovery of the Gag-filamin A interaction may provide a new therapeutic target for the treatment of HIV infection.

The HIV-1 Gag polyprotein precursor, Pr55Gag, plays a central role in virus assembly and release (1, 2). In the absence of other viral components, Gag protein itself is sufficient to generate extracellular virus-like particles (VLPs).2 The four structural domains of HIV-1 Gag, matrix (MA), capsid (CA), nucleocapsid (NC) and p6, perform distinct functions in particle assembly and budding. The MA domain is required for membrane targeting and binding. The CA domain promotes Gag multimerization. In addition to contributing to Gag-Gag interactions, the NC domain mediates genomic RNA packaging. Finally, the C-terminal p6 domain, containing the docking sites for the ESCRT (endosomal sorting complex required for transport) and ESCRT-associated proteins, is responsible for virus budding (3, 4).

In general, the plasma membrane (PM) is the predominant assembly site in most epithelial cell lines and T lymphocytes (5). Phosphatidylinositol 4,5-bisphosphate, a phosphoinositide concentrated primarily in the cytoplasmic leaflet of the PM, directs the PM targeting of Gag through an interaction with the MA domain, thereby leading to N-myristate exposure and promoting Gag membrane binding (6, 7). By contrast, in macrophages ultrastructural studies originally indicated that the late endosome (LE) and/or multivesicular body (MVB) is the assembly site, an observation supported by the presence of immature and mature virions within the intracellular LE/MVB compartment (8, 9). Recently, the cell-dependent model of HIV-1 assembly has been challenged by the studies showing that, even in macrophages, the PM is the major assembly site (10–12). The observed intracellular membranes in macrophages used for HIV budding are actually connected with the PM and are engulfed within the cytoplasm (11). However, the intracellular budding of HIV at “nonacidic” endosomes in macrophages was also documented (13). Although it has been proposed that the LE/MVB localization of HIV-1 Gag results from the clathrin-dependent endocytic event (12, 14), some investigators suggest that Gag trafficking to the LE/MVB compartment contributes to productive virus assembly (15). Indeed, several cellular factors involved in endosomal sorting pathways have been identified to play some roles in HIV-1 Gag trafficking and particle assembly. These factors include adaptor protein complexes (16–18), GGA (Golgi-localized γ-ear-containing, Arf binding) and Arf (ADP-ribosylation factor) proteins (19), annexin 2 (20), ubiquitin E3 ligase POSH (plenty of SH3s) (21, 22), and SOCS1 (the suppressor of cytokine signaling 1) (23).

In addition to vesicular trafficking machinery, host cytoskeleton dynamics are also modulated by HIV-1 to facilitate its intracellular trafficking (24, 25). Microtubule-based vesicle...
transport is dependent on kinesin and dynein motors and is responsible for the long-range motility, whereas the actin-based cytoskeleton is involved in short-range movements through the recruitment of myosin motors. The host cortical actin layer underneath the PM provides either a track or a diffusion barrier to facilitate directed intracellular movements. Early observations showed the colocalization of actin and HIV-1 Gag in membrane pseudopod structures where virus budding occurred, indicating that the actin cytoskeleton does play a role in HIV-1 assembly and budding (26). It has been proposed that HIV-1 Gag utilized actin microfilaments (F-actin) for efficient transport to the PM of infected cells (27). Indeed, cytochalasin D-induced dynamic alterations in the actin cytoskeleton impairs HIV-1 assembly (28). It has long been known that actin is incorporated into released HIV-1 virions through the direct interaction with the NC domain of HIV-1 Gag; however, the underlying mechanisms by which actin cytoskeleton is utilized by HIV-1 to facilitate particle assembly and budding remain to be defined (29, 30).

Cortical actin dynamics, responsible for signal transduction, protein trafficking, cell motility, and protrusion, is regulated by a panel of actin-binding proteins, including cross-linking, bundling, capping, and severing proteins (31). Recent studies demonstrated that cortical actin remodeling mediated by cofillin and ezrin-radixin-moesin proteins is required for the early stage of HIV infection (32, 33). However, the evidence regarding actin-binding protein-regulated actin dynamics responsible for late stages of HIV infection, including virus assembly and budding, is quite sparse.

Here, we report a novel protein-protein interaction between the actin cross-linking protein filamin A (FLNa) and HIV-1 Gag. FLNa is a 280-kDa non-muscle modular protein with an N-terminal actin binding domain and C-terminal 24 tandem immunoglobulin-like domains of ~96 amino acid residues (IgFLNai–24) as well as two hinge regions, H1 and H2. H1 is located between domains 15 and 16, and H2 is located between domains 23 and 24. Domain 24 is responsible for FLNa dimerization. FLNa serves as a versatile scaffold required for membrane receptor anchoring and trafficking, signal transduction, and cell-cell/cell-matrix connections (34, 35). In this report, we demonstrate that FLNa does play a role in the HIV-1 Gag subcellular trafficking pathway and that FLNa binding facilitates Gag trafficking to the PM.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—An expression plasmid (pREP4-FLNa) for full-length FLNa (amino acids 1–2647) was kindly provided by Fumihiko Nakamura (Harvard Medical School). Expression plasmids for C-terminal FLNa (FLNa-3', amino acids 2364–2647) with and without a HA tag were engineered by PCR amplification and subcloning into pcDNA3.1 (Invitrogen) or pCMV-HA vectors (Clontech), respectively. These constructs were verified by DNA sequencing. pNL4–3 proviral plasmids were obtained from Malcolm Martin (NIAID/NIH) through the NIH AIDS Reference and Reagent Program. The HIV-1 Gag and Protease expression plasmids 3–CCCCC were obtained from Hans-Georg Krausslich (University of Hamburg) (36). Codon-optimized Gag expression plasmids derived from pVRC3900 were provided by Gary Nabel (VRC/NIH) (37). A panel of truncated Gag mutations cloned into either Gal4 DNA binding domain vector pGBK-T7 (Clontech) for yeast two-hybrid assays or pGEX-2T (Amersham Biosciences) for glutathione S-transferase (GST) pulldown assays were described previously (18). Gag-GFP expression plasmids were described earlier by others (38). An expression plasmid encoding for the dominant negative dynamin K44A was kindly provided by Marc Caron (Duke University) (39). An expression plasmid encoding for N-terminal Eps15 (amino acids 1–858), which is the dominant negative variant of Eps15 lacking the ubiquitin-interacting motif, was kindly provided by Simona Polo (IFOM-IEO Campus, Milan, Italy) (40).

**Cell Lines, Transfections, and Infections**—M2 and A7 cell lines were kindly provided by Yasutaka Ohta and Thomas Stossett (Harvard Medical School). M2 cells were maintained in minimum essential medium supplemented with 8% newborn calf serum, 2% fetal calf serum, and antibiotics at 37 °C in 5% CO2. A7 cells were maintained in the same medium as M2 cells except for the additional supplement of 500 μg/ml G418. Jurkat cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum and antibiotics. Transfections were performed in M2 and A7 cells using Lipofectamine 2000 (Invitrogen). Transfections were performed in Jurkat cells using the Nucleofector kit (Amaxa) or TransIT-Jurkat Transfection Reagent (Mirus). In some experiments VSV-G pseudotyped NL4-3 virus stocks were applied to infect M2 and A7 cells for 6 h.

**Yeast Two-hybrid Assays**—Full-length codon-optimized Gag was cloned into the bait vector pGBK7 encoding the Gal4 DNA binding domain (BD) and was used to screen a HeLa cDNA library, which fused to the Gal4 activation domain (AD) vector pACT2 (Clontech). Positive clones were isolated and sequenced. According to the manufacturer’s instructions (Clontech), the individual protein-protein interaction was tested by one-on-one transformation. The β-galactosidase assay has been described previously (18).

**GST Pulldown**—GST fusion proteins were expressed in Escherichia coli BL21 (DE3) cells (Stratagene) through the induction of 0.1 mm isopropyl-β-D-thiogalactopyranoside (Sigma). The bacteria in lysis buffer (50 mm Tris-HCl, pH 7.6, 50 mm NaCl, 5 mm MgCl2, 1 mm DTT, 1 mm PMSF) were sonicated and precleared by centrifugation at 10,000 × g for 10 min. The samples were incubated with glutathione-Sepharose beads (GE Healthcare) to purify GST fusion proteins. The immobilized GST fusion proteins were incubated with 293T cell lysates at 4 °C for 2–4 h and washed extensively followed by immunoblotting for FLNa. Monoclonal anti-FLNa antibodies were obtained from Chemicon.

**Coimmunoprecipitation**—293T cells grown in 10-cm2 culture dishes were transfected by calcium phosphate or polyethyleneimine (Sigma) methods. Transfected cells were harvested at 40–48 h after transfection, washed with PBS buffer, and lysed with radioimmunoprecipitation assay buffer (50 mm Tris-HCl, pH 7.5, 105 mm NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 2 mm EDTA). Cell lysates were centrifuged at low speed for 10–15 min to remove the nuclei, incubated with protein A/G-Sepharose beads (Pierce) at 4 °C for 1 h, and centrifuged to remove protein A/G-Sepharose beads.
Involvement of FLNa in HIV-1 Gag Trafficking

Finally, the samples were immunoprecipitated with the indicated antibodies as well as protein A/G-Sepharose beads at 4 °C overnight and washed extensively with radioimmunoprecipitation assay buffer followed by immunoblotting for Myc, FLNa, or HIV-1 p24CA.

RNA Interference—Twenty-one nucleotide siRNA duplexes against FLNA gene with two nucleotide 3’-UU overhangs were purchased from Dharmacon. These siRNA duplexes include siRNAFLNa1, duplex targeting coding region 2555–2573 (CCACAAAGGTCAAGTATA), siRNAFLNa2 duplex targeting coding region 2160–2178 (GCCAGGAGCTGGCGATAT), and a control siRNA duplex (sense sequence, 5’-CUCUCGCGGUAUAGCAGUUU-3’; antisense sequence, 5’-ACUGC-UUUACGCGGAGAGUU-3’). siRNA transfection was performed using Lipofectamine 2000.

Immunofluorescence Microscopy—M2, A7, and HeLa cells were grown overnight on glass coverslips in 6-well plates and transfected using Lipofectamine 2000. Transfected cells were fixed with 3.8% formaldehyde in a sodium phosphate buffer at room temperature for 10–15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% bovine serum albumin in PBS for 1 h. Then cells were immunostained with the indicated antibodies and the fluorescent conjugated antibodies. In the single-staining experiments, CD63 protein or tetraspanin CD81 was revealed by mouse anti-Lamp3 antibodies (Santa Cruz Biotechnology) or mouse anti-human CD81 antibodies (BD Pharmingen) followed by goat anti-mouse Alexa 546-conjugated antibodies (Molecular Probes). Gag staining was performed with rabbit polyclonal anti-p17 antisera followed by goat anti-rabbit Alexa 546-conjugated antibodies (Molecular Probes). In double-staining experiments, HA-FLNa-3’ or FLNa was stained with mouse anti-HA (Covance) or mouse anti-FLNa (Chemicon) antibodies followed by goat anti-mouse Alexa 546-conjugated antibodies. Gag was detected by rabbit polyclonal anti-p17 antiserum followed by Alexa 488-conjugated goat anti-rabbit antibodies (Molecular Probes). Confocal images were acquired using a Nikon TE2000-U laser-scanning confocal microscope, and data analysis was performed with EZ-C1 and NIS-Elements AR software.

Flow Cytometric Analysis—HIV-1-infected Jurkat cells were fixed, permeabilized, and stained in preparation for flow cytometric analysis. Mouse anti-human p24 monoclonal antibodies (Chemicon) and rabbit polyclonal anti-FLNa antibodies (Abcam) were used for the intracellular HIV-1 p24CA and FLNa staining. The samples were run on the BD FACSCalibur flow cytometer, and the data were analyzed by Flowjo software.

Purification ofVirions—293T cells were cotransfected with siRNA and pNL4-3 proviral plasmids, or M2 and A7 cells were infected with VSV-G-pseudotyped NL4-3 viruses. Supernatants were harvested at 48 h after transfection or infection, filtered through a 0.45-μm filter, and clarified by low speed centrifugation at 3000 rpm for 10 min at 4 °C. Finally, virions were concentrated through a 20% sucrose cushion by centrifugation at 28,000 × g for 3 h at 4 °C.

Transmission Electron Microscopy—M2 and A7 cells were cotransfected with pNL4-3 proviral plasmids and empty pcDNA3.1 vectors or DN-Eps15 or DN-dynamin expression plasmids. Three independent experiments were performed. At 40–48 h after transfection, the cells in three independent experiments were harvested and pooled. The samples were fixed with 2% glutaraldehyde in phosphate buffer for overnight, post-fixed with 1% osmium tetroxide for 2 h, stained en bloc with 1% uranyl acetate for 1 h, dehydrated with an ethanol series, and embedded in araldite. Ultrathin sections were mounted on Formvar-coated grids and examined on a Phillips CM10 transmission electron microscope equipped with an AMT 2 mega pixel camera.

RESULTS

HIV-1 Gag Binds Specifically to FLNa—Host proteins cooperate with HIV-1 Gag and participate in the distinct stages of late stages of the HIV-1 replication cycle (42). To identify potential cellular proteins involved in particle assembly and release, we performed a yeast two-hybrid screen against a human cDNA library using full-length HIV-1 Gag as bait. We identified several unreported Gag-interacting proteins in addition to the previously identified Tsg101, cyclophilin B, and the δ subunit of the adaptor protein complex 3 (18, 43–45). Among those unreported Gag binding partners, FLNa was chosen for further evaluation because recent studies show that FLNa plays important roles in coupling protein trafficking with cortical actin dynamics (Fig. 1A) (46–55). The isolated cDNA clone of FLNa from our screen encodes the C terminus of FLNa representing amino acids 2364–2647, designated FLNa-3’. FLNa-3’ corresponds to the C-terminal portion of IgFLNa22, IgFLNa23, H2, and IgFLNa24 (Fig. 1A). First, we confirmed the interaction between HIV-1 Gag and FLNa-3’ by one-on-one transformation experiments (Fig. 1B). To identify the FLNa interacting region within HIV-1 Gag, we next used the yeast two hybrid approach to test for binding characteristics of the major cleavage products of Gag (MA, CA, NC, and p6) and a panel of truncated Gag mutations (MACA, MACANC, and CANCp6) to FLNa-3’. The Gal4 BD fusion proteins bearing the CA domain maintained the binding ability to FLNa-3’ fused with the Gal4 AD (Fig. 1C). The observed interactions were validated by testing the interactions of these bait fusion proteins with empty prey vector (supplemental Fig. 1). To further investigate the extent of these yeast two-hybrid interactions, we measured the β-galactosidase activity in liquid cultures. The quantitative results demonstrate that the CA domain of HIV-1 Gag is required for the FLNa-3’ binding (Fig. 1D).

The FLNa-Gag interaction was verified using a GST pull-down assay. In these experiments, a panel of recombinant GST fusion proteins representing the major domains of Gag was expressed in E. coli and purified on glutathione-Sepharose beads (Fig. 1E, top). Immobilized GST fusion proteins were then incu-
Involvement of FLNa in HIV-1 Gag Trafficking

AUGUST 12, 2011 • VOLUME 286 • NUMBER 32 • JOURNAL OF BIOLOGICAL CHEMISTRY 28501

Bated with 293T cell lysates to evaluate their abilities to bind to endogenous FLNa. Only GST-CA and GST-Gag fusion proteins, rather than other GST fusion proteins, coprecipitated efficiently with endogenous FLNa (Fig. 1E, bottom). In our experiments, a 190-kDa cleavage fragment of FLNa was observed to coprecipitate with the GST-Gag fusion protein (Fig. 1E, asterisk).

FIGURE 1. HIV-1 Gag interacts with FLNa. A, shown is a schematic representation of FLNa dimer and domain structure. aa, amino acids. B, shown is a yeast two-hybrid analysis of the Gag-FLNa interaction. The interaction between the Gal4 DNA-BD fusion protein bearing full-length Gag and the Gal4 AD fusion protein containing FLNa-3′ was tested for growth either on SD solid media lacking leucine and tryptophan (SD/-Trp/-Leu) or on SD solid media lacking adenosine, histidine, leucine, and tryptophan but containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; SD/-Ade/-His/-Trp/-Leu/X-gal). Positive or negative interaction is indicated by + or −, respectively. Three negative controls and a positive control were included. C, the interactions between different Gal4 DNA-BD fusion proteins (left) and AD-FLNa-3′ were tested for growth on selective SD solid media (right). D, the yeast two-hybrid interactions were qualified by liquid β-galactosidase assays. Three negative controls and one positive control were included. Data from three independent experiments are shown as the means ± S.D. E, GST pulldown analysis of the Gag-FLNa interaction is shown. A series of recombinant Gag proteins fused with a GST tag was expressed in E. coli and purified using glutathione-Sepharose beads. Purified GST fusion proteins were separated by SDS-PAGE and visualized by Coomassie Blue staining (top). Bead-bound GST fusion proteins were incubated with cell lysates of 293T cells followed by immunoblotting (WB) for FLNa (bottom). A 190-kDa fragment of FLNa is indicated in GST-Gag lane by an asterisk. F, coimmunoprecipitation of Gag and endogenous FLNa was shown. 10 μg of either Gag expression plasmids or empty pcDNA3.1 vectors were transfected into 293T cells. Gag protein was immunoprecipitated (IP) using HIV Ig. Coprecipitated FLNa was detected by immunoblotting with anti-FLNa antibodies (top). Western blot analysis of the precipitated Gag protein is shown using anti-p24CA antibodies (middle). Cell lysates before immunoprecipitation were probed with anti-FLNa antibodies (bottom). G, reciprocal coimmunoprecipitation of Gag and endogenous FLNa is shown. Cell lysates prepared as in F were subjected to anti-FLNa antibody immunoprecipitation and probed with anti-CA (top) and anti-FLNa (middle) antibodies. Cell lysates before immunoprecipitation were probed with anti-CA antibodies (bottom). H, coimmunoprecipitation of MACA-Myc and FLNa is shown. FLNa-F together with either MA-myc or MACA-myc was coexpressed in 293T cells. Cells lysates were immunoprecipitated with anti-FLNa antibodies followed by the detection with anti-Myc (top) and anti-FLNa (middle) antibodies. Cell lysates before immunoprecipitation were probed with anti-Myc antibodies and probed with anti-FLNa (top) and anti-Myc (middle) antibodies. Cell lysates before immunoprecipitation were probed with anti-FLNa antibodies (bottom).
Involvement of FLNa in HIV-1 Gag Trafficking

We next determined whether Gag and FLNa could coprecipitate in human cells. To this end, empty pcDNA3.1 vectors or Gag expression plasmids were transfected into 293T cells. Immunoprecipitation of Gag revealed an efficient interaction with the endogenous FLNa (Fig. 1F, top). Using reciprocal coimmunoprecipitation, we also confirmed the interaction of Gag with FLNa (Fig. 1G, top). To define the role of the CA domain in the Gag-FLNa interaction, full-length FLNa (FLNa-F) was expressed in 293T cells together with either an NCp6-deleted Gag mutant bearing a C-terminal myc epitope tag (MACA-Myc) or a C-terminal myc-tagged MA (MA-Myc). Immunoprecipitation of FLNa demonstrated a significant interaction with MACA-Myc and not with MA-Myc, as determined by Western blot analysis using anti-Myc antibodies (Fig. 1H, top). Similarly, reciprocal immunoprecipitation was performed and demonstrated the coimmunoprecipitation of FLNa with MACA-Myc (Fig. 1I). Taken together, these results reveal that HIV-1 Gag interacts efficiently with FLNa through the CA domain.

To determine whether Gag colocalizes with endogenous FLNa in human cells, we performed confocal immunofluorescence microscopy in HeLa cells. In cells without Gag expression, FLNa labeling was mainly detected in the cortical cytoplasm and PM, consistent with the role for FLNa in regulating cortical actin dynamics and anchoring membrane proteins to the PM (Fig. 2A (34)). Then we examined the colocalization of endogenous FLNa and exogenously expressed Gag. The degree of the FLNa-Gag colocalization was quantified in 15–20 transfected cells using the Pearson correlation coefficient (R). First, we evaluated the colocalization of endogenous FLNa with Gag expressed alone. At 20–24 h after transfection, Gag displayed a PM distribution pattern and overlapped with FLNa at the discrete region of the PM (Fig. 2B, top, R = 0.94 ± 0.03). Second, we observed the colocalization of endogenous FLNa with Gag expressed in the context of infectious molecular clone pNL4-3. At 20–24 h after transfection, Gag was present in discrete puncta at the PM and showed a high level of colocalization with FLNa (Fig. 2B, bottom, R = 0.89 ± 0.05). These confocal microscopic studies reveal that Gag colocalizes with endogenous FLNa in human cells.

FLNa Expression Level Correlates with HIV-1 Biogenesis—To determine whether HIV-1 infection modulates cellular FLNa protein abundance, the levels of cellular FLNa in Jurkat T cells before and after VSV-G pseudotyped HIV-1NL4-3 infection were quantified. Uninfected Jurkat T cells were included as a negative control to demonstrate the shift in FLNa staining (Fig. 3A). Flow cytometric analysis showed that HIV-1 infection upregulated cellular FLNa protein abundance (Fig. 3B). Note that there was a definite shift to the upper right quadrant. FLNa protein abundance was up-regulated by more than 10-fold in p24+ Jurkat cells, as compared with p24− cells (Fig. 3C). Western blot analysis of endogenous FLNa levels was also performed, confirming that HIV-1 infection increases FLNa expression (Fig. 3D, top).

Cellular FLNa protein abundance changes in HIV-1-infected cells may reflect that FLNa plays some roles in HIV-1 biogenesis. To this end, FLNa-F was transiently expressed in Jurkat cells, and its effect on HIV-1 replication was evaluated. In our experiments, JRCSF proviral plasmids together with an equal amount of FLNa-F expression plasmids or empty pcDNA3.1 vectors were cotransfected into non-activated Jurkat T cells or activated Jurkat T cells by phorbol 12-myristate 13-acetate plus ionomycin. Overexpression of FLNa in non-activated or activated Jurkat T cells promoted virus replication by about 3-fold as compared with control cells, which was determined by measuring the released p24 antigen (Fig. 3E). The effect of FLNa overexpression on virus replication was also evaluated in HeLa cells. pNL4-3 proviral plasmids was transfected into HeLa cells at a ratio of 1:1 with either FLNa-F expression plasmids or empty pcDNA3.1 vectors. Cellular levels of FLNa in cells receiving FLNa-F expression plasmids were enhanced by 30%, as compared with cells receiving empty pcDNA3.1 vectors (Fig. 3F, top). FLNa overexpression increased the yield of extracellular virus particles by more than 50% (Fig. 3F, bottom). However, overexpression of FLNa did not change cell-associated p24 antigen levels, supporting a role for FLNa in virus assembly and release (Fig. 3F, bottom). Similarly, an increase in Gag VLP release associated with FLNa overexpression was also observed.
in HeLa cells cotransfected with Gag-Protease and FLNa-F expression plasmids (Fig. 3G). These results suggest that FLNa plays a positive role in virus release through its interaction with HIV-1 Gag.

**Depletion of FLNa Inhibits HIV Particle Release**—To establish the biological relevance of the FLNa-Gag interaction, we depleted endogenous FLNa from HeLa cells using the short interfering RNA (siRNA) approach. Two different siRNA duplexes (siRNAFLNa1 and siRNAFLNa2) designed to target different coding regions of FLNA were transfected individually into HeLa cells. As assessed by Western blot analysis using anti-FLNa antibodies, FLNa levels in siRNAFLNa1-treated or siRNAFLNa2-treated cells were reduced by more than 60% as compared with siRNAcontrol-treated cells (data not shown). We then investigated HIV-1 particle assembly and release from siRNA-treated cells. To achieve a highly significant decrease in
Involvement of FLNa in HIV-1 Gag Trafficking

FLNa levels, siRNA was transfected into HeLa cells followed by a second transfection with siRNA as well as pNL4-3 proviral plasmids at 24 h after the first transfection. Western blot analysis was performed at 48 h after the second transfection to evaluate p24 antigen levels in cell lysates and supernatants. Virus release was inhibited by about 50% in FLNa knockdown cells as compared with control cells (Fig. 4A). p24 antigen ELISA analyses of cell lysates and supernatants in the similar experiments revealed that virus release from FLNa knockdown cells was decreased by more than 60% as compared with control cells, consistent with Western blot results (Fig. 4B).

An FLNa-depleted human melanoma cell line (M2) and a derivative rescued subline (A7) expressing approximately wild type levels of FLNa provide a model system to examine the role of FLNa in HIV-1 particle release (56). M2 and A7 cells were infected with VSV-G-pseudotyped HIV-1 NL4-3 viruses. Virus release from FLNa-depleted M2 cells was markedly diminished. However, virus release from FLNa-repleted A7 cells was very robust, as determined by Western blot analysis using HIV Ig (Fig. 4C). The results that M2 cells showed poor Gag VLP production as compared with A7 cells confirmed that FLNa facilitates HIV-1 release specifically through the Gag proteins (Fig. 4D). To determine the specific role for FLNa in HIV-1 particle assembly and release, we performed rescue experiments in FLNa-depleted M2 cells. Full-length FLNa expression plasmids were cotransfected with pNL4-3 proviral plasmids into M2 cells, and particle release was measured by p24 antigen ELISA at 48 h after transfection. The transient expression of full-length FLNa in M2 cells restored particle release (Fig. 4E). Collectively, these studies demonstrate that FLNa plays a role in HIV particle assembly and release.

FLNa Is Involved in Gag Trafficking—To define the stage of the block to HIV particle release introduced by FLNa depletion, we performed confocal microscopic studies to observe Gag-GFP localization in M2 and A7 cells at 20–24 h after transfection. A PM punctate distribution pattern of Gag-GFP was observed in more than 70% of A7 cells (Fig. 5A). Gag-GFP accumulation at the distinct region of the PM was confirmed by our colocalization studies showing that Gag-GFP colocalized with endogenous CD81 at the PM (supplemental Fig. 2). Tetraspanin CD81 is a component of tetraspanin-enriched microdomains at the PM (57). In less than 30% of A7 cells, Gag-GFP was found to be concentrated at both the PM and intracellular compartments. In these cells expressing Gag-GFP, the levels of fluorescence intensity detected at the PM were comparable with that observed at intracellular compartments (data not shown). In contrast, Gag-GFP distribution in most M2 cells showed a punctate intracellular distribution that differed markedly from what was seen in most A7 cells (Fig. 5B). The two different distribution patterns of untagged wild type Gag in A7 and M2 cells, as analyzed by immunostaining using anti-p17MA antibodies, were similar to that seen with Gag-GFP (Fig. 5, C and D). To better understand the difference in Gag subcellular localization occurred in M2 and A7 cells, we quantified wild type Gag distribution patterns in these two cell lines at two time points after transfection by counting the number of Gag-expressing cells based on whether the Gag proteins accumulated at the PM or at both the PM and intracellular compartments. At 24 h after transfection, about 64% of A7 cells exhibited a PM distribution pattern of Gag, whereas only 29% of M2 cells showed a PM distribution pattern of Gag (Fig. 5E). Similarly, at 48 h after transfection, Gag proteins in 75% of A7 cells localized to the PM. In contrast, Gag accumulation at the PM was only detected in 38% of M2 cells (Fig. 5E). These results

FIGURE 4. Depletion of FLNa inhibits HIV-1 particle assembly and release. A, HeLa cells were first transfected with 100 nm FLNa-specific siRNAfln1 (lane 2) or siRNAfln2 (lane 3) or control siRNA (siRNAcontrol, lane 1) followed by a second cotransfection with 100 nm siRNA duplex and 2 μg of pNL4-3 proviral plasmids at 24 h later. At 48 h after the second transfection, Western blot analyses of cell lysates (anti-FLNa, anti-actin, and anti-p24) and pelleted virion lysates (anti-p24) were performed. B, siRNA experiments were performed in a similar manner as described in A. p24 levels in cell lysates (top) and supernatants (bottom) were measured by ELISA assays (ns, no significance; **, p < 0.01). Results represent the mean ± S.D. from three independent transfections. C, M2 and A7 cells were infected with VSV-G-pseudotyped NL4-3 viruses. At 40–48 h post-infection, Western blot analyses of cell lysates (anti-FLNa, anti-actin, and anti-p24) and pelleted virion lysates (anti-p24) were performed. D, M2 and A7 cells were transfected with 2 μg of Gag-Pr (top) or Gag expression plasmids (bottom), p24 levels in the cell lysates and supernatants were measured at 40–48 h after transfection. Results shown represent the mean ± S.D. (n = 3). E, M2 cells were cotransfected with 1 μg of pNL4-3 proviral plasmids and 1 μg of either empty pcDNA3.1 vectors or FLNa-F expression plasmids. At 40–48 h after transfection, Western blot analyses of cell lysates (with anti-FLNa or anti-actin antibodies) were performed (top and middle). p24 antigen in the cell lysates and supernatants (bottom) was quantified by ELISA assays (***, p < 0.001). Data represent the mean ± S.D. from triplicate transfections.
suggest that FLNa depletion reduces localization of Gag to the PM. To define the specificity of the FLNa role in the subcellular localization of HIV-1 Gag, full-length FLNa was expressed transiently in M2 cells and HIV-1 Gag subcellular localization was examined. The detection of Gag and FLNa was performed by immunostaining with mouse anti-FLNa antibodies and rabbit polyclonal anti-p17 antisera, respectively. Transient expression of FLNa in M2 cells reversed the intracellular distribution pattern of Gag in M2 cells and restored the high level of PM localization of Gag proteins normally seen in FLNa-repleted A7 cells at 20–24 h after transfection (Fig. 5F). In M2 cells with exogenous FLNa expression, HIV-1 Gag was present in discrete

**FIGURE 5.** Depletion of FLNa induces HIV-1 Gag accumulation at internal compartments. A and B, confocal analysis of A7 (A) and M2 (B) cells transfected with 2 μg of Gag-GFP expression plasmids is shown. Gag-GFP is shown in green (left). The DIC images are shown on the right. Scale bars represent 10 μm. C and D, immunofluorescence staining of HIV-1 p17MA in A7 (C) and M2 (D) cells transfected with 2 μg of Gag expression plasmids is shown. Gag is shown in red (left). The DIC images are shown on the right. Scale bars represent 10 μm. E, quantitation of Gag subcellular localization is shown. M2 and A7 cells were transfected with 2 μg of Gag expression plasmids. At 24 or 48 h after transfection, cells were fixed for immunostaining using anti-p17 antisera. The ratios of the number of cells with Gag accumulation at the PM to the number of cells with Gag location at both intracellular compartments and the PM (Int. + PM) were quantified. 50 cells were counted to represent the result. Two independent experiments were performed. F, M2 cells were cotransfected with 1 μg of FLNa-F and Gag expression plasmids. At 20–24 h after transfection, cells were immunostained to detect FLNa and Gag. FLNa is shown in red (left), Gag in green (center), and colocalized pixels in yellow (right). Scale bars represent 10 μm. G, M2 (top) and A7 cells (bottom) were transfected with 2 μg of Gag-GFP expression plasmids. After 24 h, cells were fixed, permeabilized, and immunostained using anti-CD63 antibodies followed by anti-mouse Alexa 546-conjugated antibodies. Gag-GFP is shown in green (far left panel), CD63 in red (left panel), and the colocalized pixels in yellow (right panel). The DIC images merged with confocal images of Gag-GFP and CD63 are shown in the far right panel. Scale bars represent 10 μm. The R value was measured in 15–20 transfected cells. Three independent experiments were performed.
Involvement of FLNa in HIV-1 Gag Trafficking

puncta at the PM and colocalized with transiently expressed FLNa (Fig. 5F, right). These results are consistent with FLNa contributing to the subcellular localization of HIV-1 Gag.

To determine the nature of the intracellular vesicle-like structures where Gag is enriched in FLNa-depleted cells, we performed colocalization studies. HIV-1 Gag has been shown to traffic to the LE/MVB compartment in several cell lines (18). Therefore, we examined whether the observed intracellular vesicular structures in M2 cells where Gag accumulates are LE/MVB compartments. To this end, CD63-HcRed and Gag-GFP expression plasmids were cotransfected into M2 and A7 cells. M2 cells, but not A7 cells, showed a marked colocalization of these two fluorescent proteins at the perinuclear region (supplemental Fig. 3). When Gag-GFP-expressed M2 cells were immunostained for endogenous CD63, a significant Gag-GFP and CD63 colocalization was observed (Fig. 5G, top, R = 0.73 ± 0.05). By contrast, the Gag-GFP and CD63 intracellular colocalization did not appear in A7 cells (Fig. 5G, bottom, R = 0.11 ± 0.02). In these A7 cells, Gag-GFP localized primarily to the PM (Fig. 5G, bottom). Taken together, these data suggest that FLNa depletion induces Gag accumulation at the LE/MVB compartment.

A C-terminal Fragment of FLNa Inhibits HIV-1 Particle Release in a Dose-dependent Manner—FLNa-3’, a C-terminal fragment of FLNa, was identified to interact with the CA domain of HIV-1 Gag in the yeast two-hybrid-based analysis (Fig. 1B). We reasoned that FLNa-3’ expression in mammalian cells could sequester Gag from cellular FLNa, resulting in a reduction in the yield of extracellular virus particles. To determine the effect of FLNa-3’ on particle release, FLNa-3’ expression plasmids together with pNL4-3 proviral plasmids were transfected into HeLa cells at different ratios, and virus release was evaluated at 40–48 h after co-transfection. As expected, FLNa-3’ inhibited virus release in a dose-dependent manner (data not shown). To facilitate detection of FLNa-3’ expression, we constructed an HA-tagged FLNa-3’ expression plasmid and examined its effect on virus release. The enhanced expression of HA-FLNa-3’ inhibited progressively virus release (Fig. 6A, bottom). By contrast, the cellular levels of HIV-1 Gag and actin were comparable in cells with different doses of HA-FLNa-3’, excluding the possibility of FLNa-3’-induced global cytotoxicity (Fig. 6A, top three panels). These virological studies confirm the role of FLNa in virus assembly and release. The dose-dependent inhibition of particle release induced by FLNa-3’ was also observed in Jurkat T cells cotransfected with pNL4-3 proviral plasmids and FLNa-3’ expression plasmids at different ratios from 1:0 to 1:3 (Fig. 6B).

Dominant Inhibition of the Gag-FLNa Interaction Induces Gag Accumulation within the Internal Compartments—To determine the effect of FLNa-3’ in Gag trafficking, we compared Gag-GFP subcellular distribution pattern in HeLa cells in the presence versus absence of FLNa-3’. At 20–24 h after transfection, Gag-GFP exhibited a predominantly punctate PM distribution pattern in cells without FLNa-3’ expression (Fig. 6C, top). By contrast, in cells with FLNa-3’ expression, Gag-GFP displayed an intracellular punctate distribution pattern, characterized by accumulation at intracellular vesicle-like structures (Fig. 6C, bottom). The effect of HA-tagged FLNa-3’ on the subcellular distribution of Gag was also evaluated. At 20–24 h after transfection, Gag localized predominantly at the PM in cells without HA-FLNa-3’ expression, whereas HA-FLNa-3’ expression eliminated Gag localization at the PM and induced Gag accumulation at intracellular compartments (Fig. 6D). The two different distribution patterns of Gag proteins in cells with or without FLNa-3’ expression reveal that FLNa-3’ interferes with the Gag trafficking route, resulting in the inhibition of particle production.

Blocking Clathrin-dependent Endocytosis in FLNa-depleted Cells Does Not Rescue Particle Release—Recent studies suggest that the LE/MVB localization of HIV-1 Gag results from virion or unassembled Gag endocytosis from the PM (12, 14). The observed LE/MVB localization of HIV-1 Gag in FLNa-depleted M2 cells raises the possibility that the role for FLNa in Gag trafficking is to inhibit the endocytosis of Gag at the PM. Under this possibility, FLNa depletion could promote an endocytic event of Gag from the PM, resulting in the inhibition of particle release. Thus, blocking endocytic pathways in FLNa-depleted cells could relieve the restriction to particle release. To test this hypothesis, we utilized a dominant-negative (DN) approach to disrupt specific stages in the endocytic pathway. Dynamin is a large GTPase required for clathrin-coated vesicle budding from the PM, and epidermal growth factor receptor substrate 15 (Eps15) is involved in clathrin-coated vesicle formation at the PM through interactions with other clathrin adaptor proteins such as adaptor protein complex 2 (58, 59). Expression of either the dynamin K44A mutant or the ubiquitin interacting motif-deleted Eps15 mutant specifically blocks clathrin-dependent endocytic events in a dominant-negative fashion (60). Indeed, expression of either DN-dynamin (Fig. 7A, middle) or DN-Eps15 (Fig. 7A, bottom) in M2 cells efficiently inhibited the clathrin-mediated uptake of transferrin. We examined the effect of the two DN mutants on particle release. HIV-1 Gag-Protease was coexpressed with either DN-dynamin or DN-Eps15 in FLNa-depleted M2 cells or FLNa-repleted A7 cells. Blocking clathrin-dependent endocytosis did not rescue Gag VLP release from M2 cells (Fig. 7B, bottom). Also, neither DN-Eps15 nor DN-dynamin expression in M2 cells relieved the restriction to HIV-1NL4-3 virus release (Fig. 7C). We performed transmission electron microscopic studies in these transfected cells and calculated the average number of budding and released particles from the surface of about 50–80 pNL4-3-cotransfected cells with empty pcDNA3.1 vectors or DN-Eps15 or DN-dynamin expression plasmids. 30 budding and cell-free particles surrounding the surface of each NL4-3-transfected A7 cells were observed (Fig. 7D, top left). In contrast, only six budding and released particles on the cell surface per pNL4-3-transfected M2 cell were detected (Fig. 7D, top right). Expression of neither DN-Eps15 (Fig. 7D, bottom left) nor DN-dynamin (Fig. 7D, bottom right) in M2 cells significantly changed the average number of budding and released particles on the surface per transfected cell, as the average number for these different experiments was 5 and 8, respectively. We also observed Gag-GFP localization pattern in M2 cells in the presence of endocytosis inhibitors. Gag-GFP in M2 cells expressing either DN-dynamin (Fig. 7E, bottom right) or DN-Eps15 (Fig. 7E, top right) predominantly exhibited the intracellular punc-
Involvement of FLNa in HIV-1 Gag Trafficking

In this report we demonstrate that HIV-1 Gag binds to actin-binding protein FLNa and colocalizes with FLNa at the distinct region of the PM. FLNa depletion or dominant inhibition of the Gag-FLNa interaction inhibited particle release and eliminated Gag location at the PM. By contrast, FLNa repletion increased particle release and promoted Gag location at the PM. Moreover, FLNa-mediated trafficking of Gag is independent of clathrin-mediated endocytic events. Our data, therefore, suggest that the FLNa binding facilitates HIV-1 Gag trafficking to the PM.

DISCUSSION

In this report we demonstrate that HIV-1 Gag binds to actin-binding protein FLNa and colocalizes with FLNa at the distinct region of the PM. FLNa depletion or dominant inhibition of the Gag-FLNa interaction inhibited particle release and eliminated Gag location at the PM. By contrast, FLNa repletion increased particle release and promoted Gag location at the PM. Moreover, FLNa-mediated trafficking of Gag is independent of clathrin-mediated endocytic events. Our data, therefore, suggest that the FLNa binding facilitates HIV-1 Gag trafficking to the PM.

Our flow cytometric analysis and Western blot results indicated an increase in FLNa abundance in Jurkat T cells upon HIV-1 infection (Fig. 3, C and D). These data are consistent with the previous large-scale quantitative analysis of a HIV-1LAI-infected CD4+ T cell line showing that FLNa was significantly increased in abundance in CEM×174 cells upon acute HIV-1 infection (61). In those experiments FLNa was identified as 1 of the top 34 up-regulated signature proteins (61). FLNa abundance alternations in human primary CD4+ T cells upon ex vivo HIV-1 infection and in peripheral blood mononuclear cells isolated from HIV-1-positive patients was also reported recently.
All these studies suggest that FLNa is closely related to HIV-1 biogenesis. Indeed, overexpression of FLNa in Jurkat cells or phorbol 12-myristate 13-acetate/ionomycin-activated Jurkat cells promoted HIV-1JRCSF replication (Fig. 3E). Moreover, an increase in the yield of extracellular virions or VLPs induced by FLNa overexpression was not due to the enhanced HIV-1 Gag expression, consistent with our hypothesis that FLNa is involved in virus assembly and release (Fig. 3, F and G).

The proteomic analysis of monocyte-derived macrophage-derived HIV-1 virions, which were highly purified by density centrifugation and CD45 immunoaffinity depletion, indicates that FLNa is incorporated into virions (64, 65). To date, the exact mechanism of FLNa incorporation remains to be defined; however, our binding analyses suggest that the Gag-FLNa interaction might mediate FLNa incorporation (Fig. 1). FLNa incorporation into virions as well as FLNa colocalization (62, 63).
with Gag at distinct regions of the PM implies that FLNa acts as a partner in the assembly and budding process (Fig. 2B). Indeed, FLNa depletion or dominant inhibition of the Gag–FLNa interaction eliminated Gag localization at the PM (Figs. 5, B and D, and 6, C and D). Inversely, FLNa repletion in FLNa-depleted cells restored the PM localization of Gag (Fig. 5, A, C, and F). These loss-of-function and gain-of-function experiments suggest that the FLNa-Gag interaction contributes to the PM localization of Gag.

The structural properties of FLNa enable its scaffolding function in cell motility and signaling, transcription regulation, and organ development (34, 66–69). Recently, several lines of evidence support a role for FLNas in regulating the trafficking of a variety of membrane proteins in and out of the PM through exocytic and endocytic pathways. These membrane proteins include ion channels (46, 47), receptors (48–53), glycoprotein GP1bα (54), furin (55), and caveolin-1 (70). Thus, the two distinct distribution patterns of Gag proteins, which were observed in cells with versus without FLNa and in cells with versus without FLNa-3’, suggest that the FLNa binding may either promote Gag trafficking to the PM or inhibit Gag endocytosis from the PM. Based on the experiments using a dominant negative approach to block clathrin-mediated endocytosis, we favor the first model because expression of either DN-dynamin or DN-Eps15 in FLNa-depleted M2 cells did not rescue particle release (Fig. 7, B–D) or reverse Gag location from intracellular compartments to the PM (Fig. 7E). In the current study we sought to identify the subcellular site where HIV-1 Gag recruits FLNa. Our colocalization studies demonstrate that FLNa depletion was associated with Gag accumulation at LE/MVB compartments (Fig. 5G and supplemental Fig. 3). These data suggest that LE/MVB compartments may be sites for FLNa to recognize Gag. If this is correct, the FLNa protein could be involved in the controversial LE/MVB-to-PM trafficking route of HIV-1 Gag (15, 71, 72). However, we also consider an alternative possibility based on the evidence that FLNa expression is required for the correct localization of LE/MVB compartments (55). These observations support the pleiotropic roles of FLNas in organization and cellular compartments. Thus, it is not surprising that FLNa depletion could elicit a pleiotropic defect in protein trafficking and subcellular localization of LE/MVB compartments, resulting in the mistargeting of Gag at observed LE/MVB compartments (Fig. 5G and supplemental Fig. 3).

Our yeast two-hybrid assays demonstrate that the CA domain of HIV-1 Gag physically interacts with the IgFLNa22–24 fragment (FLNa-3’) (Fig. 1 and supplemental Fig. 1). Further mapping studies indicate that only IgFLNa22–23, but not IgFLNa24, is involved in the interaction (data not shown). These binding studies are consistent with the structural studies of FLNa emphasizing that IgFLNa16–23 makes major contributions to the distinct interactions with cellular partners of great functional diversity (73). Of note, the earlier studies showed an interaction between the NC domain of HIV-1 Gag and actin cytoskeleton, suggesting that HIV-1 Gag might utilize actin cytoskeleton for trafficking to the PM to promote assembly (27–29, 74). Consistent with these studies as well as the well-defined role of FLNa in modulating the dynamics of actin cytoskeleton underneath the PM, we propose a model for FLNa function in facilitating Gag trafficking to the PM (Fig. 8). Under such a model, newly synthesized Gag is first transported to the cortical actin layer where FLNa recognizes Gag. Then, binding of Gag to FLNa induces the reorganization of actin cytoskeleton underneath the PM, promoting Gag move toward the PM along actin filaments.

In summary, we have shown that FLNas is involved in the Gag trafficking pathway in a productive manner. Our results link actin cytoskeleton dynamics to Gag intracellular trafficking events. FLNa-mediated trafficking of Gag may represent a new therapeutic target for the development of HIV/AIDS treatments.

Acknowledgments—We thank Alexandre Benmerah, Marc Caron, Hans-Georg Krausslich, Fumihiko Nakamura, Yasutaka Ohta, Simona Polo, and Tomas Stossel for reagents. HIV Ig was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program. We thank Meharry Morphology Core for assistance with confocal microscopy. We thank Qiuqiu Shao for assistance with flow cytometry.

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