Intracellular Transport of Human Immunodeficiency Virus Type 1 Genomic RNA and Viral Production Are Dependent on Dynein Motor Function and Late Endosome Positioning

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Our earlier work indicated that the human immunodeficiency virus type 1 (HIV-1) genomic RNA (vRNA) is trafficked to the microtubule-organizing center (MTOC) when heterogeneous nuclear ribonucleoprotein A2/B1 is depleted from cells. Also, Rab7-interacting lysosomal protein promoted dynein motor complex, late endosome and vRNA clustering at the MTOC suggesting that the dynein motor and late endosomes were involved in vRNA trafficking. To investigate the role of the dynein motor in vRNA trafficking, dynein motor function was disrupted by small interference RNA-mediated depletion of the dynein heavy chain or by p50/dynamitin overexpression. These treatments led to a marked relocalization of vRNA and viral structural protein Gag to the cell periphery with late endosomes and a severalfold increase in HIV-1 production. In contrast, rerouting vRNA to the MTOC reduced virus production. vRNA localization depended on Gag membrane association as shown using both myristoylation and Gag nucleocapsid domain proviral mutants. Furthermore, the cytoplasmic localization of vRNA and Gag was not attributable to intracellular or internalized endocytosed virus particles. Our results demonstrate that dynein motor function is important for regulating Gag and vRNA egress on endosomal membranes in the cytoplasm to directly impact on viral production.

HIV-12 gene transcription generates a 9-kb primary transcript, the genomic RNA (termed vRNA herein). vRNA can remain unspliced, but it is also singly or multiply spliced to generate over 30 additional mRNAs (1). vRNA encodes 55- and 160-kDa precursor proteins pr55Gag (termed Gag herein) and pr160Gag/Pol that are cleaved following virus assembly during virus maturation to yield both structural proteins and enzymes, which are critical for virus replication. The singly spliced, 4-kb mRNAs encode the auxiliary proteins Vpr, Vpu, and Vif, factors that enhance HIV-1 pathogenesis (2–4) as well as the integral membrane protein envelope (Env) that is found at the surface of virus particles. The multiply spliced, 2-kb mRNAs encode the regulatory proteins Tat, Rev, and Nef, proteins that act at both transcriptional and post-transcriptional levels. Although transcripts that are processed incorrectly are retained and degraded within the nucleus (5, 6), vRNA and the 4-kb mRNAs that both contain intronic sequences are exported from the nucleus to the cytoplasm via Rev and the chromosome region maintenance protein 1 nuclear export pathway (7). The well characterized Rev-mediated RNA transport is accomplished by the concerted activities of host cell factors in the nucleus, nuclear membrane, and in the cytosol (8–11).

It has been largely assumed that following transport of HIV-1 RNAs to the cytoplasm, the RNAs either diffuse to the polysomes for translation and/or diffuse to the plasma membrane for assembly. In fact, the cell cytosol is a cluttered environment requiring regulated and energy-dependent transport processes (Ref. 12, and recently reviewed for HIV-1 (13)). Host cell proteins such as human Rev-interacting protein (14, 15) and heterogeneous nuclear ribonucleoprotein A2/B1 (16, 17) act at discrete steps following the entry of HIV-1 RNA into the cytoplasm (11, 18) facilitating the transport of this and other mRNAs. Consistently, virtually all models for cytoplasmic RNA trafficking include both temporal and spatial roles for proteins and movement that is powered by motor proteins on the cytoskeleton in the cell (11, 19, 20).

A proportion of the vRNA must also be trafficked to regions of the cell where HIV-1 assembly occurs. This can be achieved, for example, by co-trafficking of viral components in a ribonucleoprotein complex in a microtubule-dependent manner, as we have previously shown (21). The vRNA may also have a targeting signal inherent in the molecule such as a cis-acting...
RNA localization sequence (18). The viral proteins may be equally targeted to virus assembly sites via membrane or nuclear localization/export targeting signals in the Gag protein (22–25) or via the activity of polarization signals that are found in the Env proteins of several retroviruses (26).

In the case of the trafficking of the genomic RNA of a simple retrovirus such as murine leukemia virus (MLV), evidence has been provided to demonstrate the dependence on early endosomal vesicle trafficking and requirements for GagMLV and Env+MLV proteins. An interaction between the nucleocapsid (NC) domain of GagMLV with the psi RNA packaging sequence was also found to be important for directed, microtubule-dependent trafficking (27, 28). These earlier data demonstrate that MLV uses cellular vesicular trafficking machinery during viral egress.

There is also substantial evidence that membrane or endosomal trafficking machineries are used for intracellular trafficking of HIV-1 components (29–37). For example, Gag multimerizes and interacts with membranes via its myristoylated N terminus, including intracellular endocytic membranes, indicating that Gag may use the host intracellular vesicular trafficking machinery to aid in the assembly of HIV-1 (37–39). In addition, recent experiments show that Gag interacts with several components of the endosomal sorting complex required for transport complexes to achieve biogenesis and budding of HIV-1 (35).

For retroviruses, the final destination of the full-length and unspliced vRNA is the assembling virus. This step is characterized by a selective interaction between both an intact NC domain of Gag and the psi RNA domain in the 5′-untranslated region of the vRNA (40), and for HIV-1, this has been shown to occur initially near the cell nucleus at the MTOC (17, 41). This event may be positively influenced by de novo synthesized Gag protein at this site (33). The selection of the RNA by Gag for encapsidation might also be coupled to the translation of the genomic RNA (17, 42–44), and this process likely requires the co-opting of host proteins such as Staufen1 that bind and transport mRNAs (45–47). The roles that we have defined for Staufen1 in vRNA translation (48), in promoting HIV-1 Gag multimerization (49) and in vRNA encapsidation (47), underscore the intimate relationship between the transport of the vRNA and HIV-1 assembly.

In earlier work, we found that the expression of the Rab7-interacting lysosomal protein (RILP) together with HIV-1 resulted in a dramatic enrichment of the vRNA at the MTOC. Because RILP also induces the clustering of late endosomal vesicles, multivesicular body, and the dynein/dynactin motor complex at the MTOC (50, 51), vRNA localization and trafficking might depend on either an active dynein motor and/or endosomal vesicle positioning. We therefore investigated the role of the dynein motor during vRNA egress in the cytoplasm. We disrupted the function of the dynein motor complex in two ways, and this led to the accumulation of vRNA and Gag at the cell periphery along with late endosomes. This was accompanied by a severalfold increase in virus production. In contrast, RILP expression led to an enrichment of vRNA at the MTOC and resulted in lowered virus yield. Gag must retain the ability to associate with membranes to observe the redistribution of Gag and vRNA to either the periphery or MTOC. The observed localization patterns of Gag and vRNA were not due to endocytosis nor to intracellular accumulations of virus particles. Our results demonstrate that vRNA localization requires both the activity of the dynein motor and endosomal membranes for vRNA egress during HIV-1 replication.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. 16 h prior to transfection, cells were trypsinized, counted, and re-plated in antibiotic-free medium.

**Transfection and siRNAs**—Transfection of plasmids and siRNAs was performed using Lipofectamine 2000 (Invitrogen) in Opti-MEM serum-reduced medium (Invitrogen), according to the manufacturer's protocol. Knockdown of the dynein heavy chain (DHC) was achieved using a double transfection method as previously described (17, 46). On day 1, cells were transfected with 10 ns of either a control, non-silencing siRNA (siNS) 5′-AAATTCTCCGAACTGTTCAGA or a DHC-specific siRNA (siDHC) 5′-GATCAACAATGCAGGAAATT based on murine siRNA described in earlier work (52). siRNA duplexes were synthesized by Qiagen-Xeragon, CA. Three other siRNA targets to the human DHC mRNA were also used to successfully deplete cells of DHC (not shown). 24 h later, cells were transfected a second time with siNS or siDHC and 1 μg of proviral DNA per 10 cm². The proviral DNA construct used in most experiments was HxBRU (Vif+, Vpr+, Vpu−, and Nef− (16, 17)). Other proviral constructs used were: SVBH10(myr−) (a proviral clone based on BH10 with the genotype Vif−, Vpr72AA, Vpu−, and Nef−; kindly provided by Lawrence Kleiman, McGill University); pNL4.3 (a proviral clone expressing the full complement of viral proteins) (16, 17); pNL4.3/Vpu− (a proviral clone expressing the full complement of viral proteins except Vpu, generously provided by Klaus Strebel, National Institutes of Health). The proviral DNAs based on pNL4.3 harboring mutations in the NC region of Gag were NC/Delta1 (in which the first NC zinc finger is deleted) (53) and NC/SSE in which the linker region between zinc fingers #1 and #2 is mutated (54). p50/dynamitin-HA (55) and pRILP-Myc (37) were generously provided by Trina Schroer (Johns Hopkins University) and Markus Thali (University of Vermont), respectively. Green fluorescent protein (GFP)-tagged dominant negative mutants of Rab5 (Rab5 DN/GFP) (56, 57) and of Eps15 (Eps15 DN/GFP) (58) were generous gifts from Robert Lodge (Université Laval, Québec) and John Presley (McGill University, Montréal). siNS (10 nm) and pcdNA3 DNA (Invitrogen) were included in transfection mixes to supplement total amounts of siRNA and DNA or were always supplemented in control transfections.

**IF and FISH**—Immunofluorescence (IF) and FISH analyses were performed exactly as described (16, 17). Briefly, at 48 h post-transfection (or at the time points indicated), medium was carefully removed, and cells containing coverslips were washed twice with PBS. They were then fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were gently washed with PBS and then permeabilized in a 0.2% Triton X-100 in PBS for 10 min at room temperature.
HIV-1 RNP Trafficking Mediated by the Dynein Motor

For three-color combined IF/FISH analyses our initial protocol was modified as follows. The digoxigenin-labeled RNA FISH probe was generated by in vitro transcription from the plasmid pKS-Pol236nt in the presence of digoxigenin-labeled UTP as described previously (16, 17). After hybridization with the digoxigenin-labeled probe, vRNA was visualized by staining with biotinylated mouse anti-digoxigenin-Mab (Sigma-Aldrich), followed by incubation with a mouse anti-biotin-Mab conjugated with AlexaFluor 488 or a donkey anti-mouse IgG conjugated with AlexaFluor 647 (Invitrogen). In the latter case, the fluorescence signal for vRNA is detected in the far red spectrum and pseudocolored blue (Fig. 6). This signal was found to be generally weaker than that obtained using the AlexaFluor 488-coupled secondary antibody, because there is no signal amplification using the secondary anti-biotin antibody. IF detection of proteins was achieved with rabbit anti-DHC (Santa Cruz Biotechnology, Santa Cruz, CA), mouse or rabbit anti-LAMP-1 (both generously provided by Minoru Fukuda, University of California), rabbit anti-Myc (US Biologicals), rabbit anti-p50/dynamitin (Chemicon International, Inc.), and rabbit anti-Staufen1 (generously provided by Graciela Boccaccio, University of California), rabbit anti-Myc (US Biologicals), rabbit LAMP-1 (both generously provided by Minoru Fukuda, University of Buenos Aires, Argentina). Gag was identified by a sheep anti-p17 polyclonal serum (from Dr. Michael Phelan and the NIH AIDS Reference and Reagent Program). Donkey AlexaFluor488-, 594- and 647-coupled secondary antibodies were purchased from Invitrogen. Laser scanning confocal microscopy was performed using a Zeiss LSM5 Pascal confocal microscope equipped with a 63× (1.4 numerical aperture, oil immersion) Plan-Apochromat objective. A multitrack laser scanning protocol was used. Scanning was performed at 1024×1024 pixel resolution. Signals from images obtained with AlexaFluor 647 were pseudocolored in blue. Co-localization coefficients were calculated using the Manders’ overlap coefficient exactly as described (17, 59), subtracting weak fluorescence signals as background. Only cells with HIV-1 expression and/or demonstrable LAMP-1 relocalization, as an indicator of siDHC or p50/dynamitin-HA expression were included in this analysis. For IF/FISH experiments, at least 30–50 cells were included in the analyses for each condition from at least three experiments, except where indicated. In the calculation of co-localization coefficients, at least 10 cells were used for calculations for each experimental condition from 2 to 3 experiments. Transferrin uptake experiments were performed in HeLa cells as described (60) using Texas Red-labeled transferrin (Trf, Invitrogen).

Immunoblotting—Western blotting was performed after separating proteins on 12% SDS-PAGE gels except for DHC, which was identified by immunoblotting of 6% SDS-PAGE gels. Immunoblotting was performed with the following antibodies: rabbit anti-γ-tubulin (1:5000, Sigma-Aldrich) and rabbit anti-GAPDH (1:5000, Sigma-Aldrich); rabbit anti-Myc (US Biologicals); mouse anti-nucleolin and rabbit anti-fibrillarin were generously provided by Chen Liang (McGill University) (61); mouse anti-Na+/K+ ATPase was described earlier (49), and mouse anti-p24 (clone 183-H12-5C was obtained from the NIH AIDS Reference and Reagent Program (62)). Horseradish peroxidase-conjugated goat anti-rabbit (1:5000, Rockland) and sheep anti-mouse (1:5000, Rockland) were used as secondary antibodies. Finally, signals were exposed by autoradiography following development with Western Lightning Chemiluminescence Reagents as described by the manufacturer (PerkinElmer Life Sciences). Quantification of the Western blots was performed using ImageJ software (NIH).

Virus Purification and the p24 ELISA—Supernatants were collected and centrifuged 30 min at 1000×g, and viruses were purified by pelleting through 20% sucrose as described (49). Viruses were gently resuspended in PBS and quantitated by a p24-ELISA as previously described (16).

Transmission Electron Microscopy—HeLa cells were grown and transfected in 65-cm² dishes, fixed at 48 h post-transfection in 2% glutaraldehyde in PBS for 1 h, washed 2 times with PBS, scraped, and pelleted at 500×g for 10 min. After a post-fixation with 1% OsO₄ in PBS for 1 h, the samples were progressively dehydrated through an ethanol series (30%, 50%, 70%, 85%, 95%, and 100% for 20 min each), followed by 100% acetone for 20 min, and embedding in Epon 812 (Fluka). Sections, 70–90 nm thick, were cut on a Leica Ultramicrotome (Leica Microsystems) using a diamond knife, collected onto Parlodion/carbon-coated copper grids, stained with 2% uranyl acetate and 2% lead citrate, and examined using a Hitachi H7600 transmission electron microscope.

In general, siDHC, p50/dynamitin-HA, and RILP-Myc treatments affected late endosome distribution in over >80–95% cells. However, for the electron microscopy analyses, additional phenotypic proof for either siDHC knockdown, p50/dynamitin-HA, or RILP-Myc expression in cells was required. siDHC treatment results in a dispersed Golgi with fragmented Golgi membranes in the cell (63). This can be observed in the images provided and was used as a hallmark indicator for DHC depletion, although the knockdown efficiencies of DHC in this set of experiments was typically >85%. When overexpressed, p50/dynamitin-HA levels reached 10-fold over endogenous levels. Nevertheless, a major hallmark of this treatment is the appearance of Golgi stacks and associated small trafficking vesicles at the cell periphery (64). The latter was used as a marker for a cell in which p50/dynamitin-HA was overexpressed. For RILP-Myc-expressing cells, we also identified cells in which late endosomes and multivesicular bodies accumulated in the vicinity of the MTOC (51). Only cells that exhibited these phenotypes and evidence for virus production (i.e. virus particles at the cell surface) were selected in the analyses. Electron microscopy analyses were performed in five independent experiments for each condition, and the images shown are representative of the phenotype observed for each condition tested with the outlined constraints indicated above.

Virus Incubations with HeLa Cells—Conditioned medium containing released virus from pNL4.3-transfected cells (at 30–48 h post-transfection) was directly incubated with 2×10⁵ fresh, adherent HeLa cells for 4–12 h as indicated. Alternatively, 200 ng of p24-equivalents of purified HIV-1 (quantitated by a p24-ELISA) was incubated with 2×10⁵ HeLa cells/well. VSV-G-pseudotyped HIV-1 was prepared in 293T cells as described previously (65) using a pNL4-3/GFP (Env−) provirus (66) and titered by a radioactive reverse transcriptase assay using radiolabeled thymidine as described previously (66). Infection of fresh HeLa cells with VSV-G-pseudotyped HIV-1
was performed at 0.1 cpm reverse transcriptase units per cell. This led to a 20–25% infection rate as determined by GFP fluorescence of fixed cells at 24 h post-infection. At various time points following virus incubation or post-infection using VSV-G-pseudotyped HIV-1, IF and FISH analyses were performed as described above to assess LAMP-1 and vRNA localization patterns.

RESULTS

The aim of this study was to investigate the role of the dynein motor in HIV-1 viral replication. The dynein motor complex is a large multiprotein complex comprising dynein heavy, intermediate, and light chains coupled to a dynactin complex (67). To block its function we either depleted the DHC by siRNA (siDHC) or overexpressed p50/dynamitin-HA from three experiments. C, LAMP-1 = endosome localization patterns in siNS-, siDHC-, p50/dynamitin-HA-, or RILP-Myc-treated cells. LAMP-1 is in green, and DHC, p50/dynamitin, and RILP-Myc are in red. Arrows show resultant accumulations of LAMP-1 in cells. The scale bar is 10 μm.

The reduction in dynein motor function by both siDHC treatment or p50/dynamitin-HA overexpression increased virus production ~3-fold. On the other hand, when RILP-Myc was overexpressed there was a 30% reduction in virus production (Fig. 1B). These results show that modulating dynein motor function affects virus production but has no effect on the level of Gag expression in cells.

The disruption of dynein motor function and its localization by RILP expression could modulate virus production by affecting directed transport of viral components. This could be achieved by promoting the trafficking of either cellular membranes or vesicles and/or ribonucleoprotein complexes that contain viral and host proteins and RNA. Apart from the effects of p50/dynamitin on the cellular localization of the trans-Golgi and aggresomes, the overexpression of both p50/dynamitin and RILP results in dramatic changes in the localization of late
endosomes in cells, clustering at either the cell periphery or MTOC, respectively (50–52, 64). We confirmed the effect of these treatments on the localization of LAMP-1-positive (LAMP-1+/H11001) late endosomal vesicles by indirect immunofluorescence. In control (siNS-treated) cells, the distribution of late endosomes was predominantly perinuclear in 95% cells (number of cells, n = 49–58 cells in a typical experiment), whereas, in siDHC-treated cells, LAMP-1 localized to the cell periphery in 90% of cells (n = 43–54 cells in a typical experiment). DHC staining was noticeably reduced in siDHC-treated cells as expected (see Fig. 1A). p50/dynamitin-HA had the same effect as the siDHC treatment. LAMP-1 staining was noticeably reduced in siDHC-treated cells as expected (see Fig. 1A). p50/dynamitin-HA had the same effect as the siDHC treatment. LAMP-1 staining was noticeably reduced in siDHC-treated cells as expected (see Fig. 1A). p50/dynamitin-HA had the same effect as the siDHC treatment. LAMP-1 staining was noticeably reduced in siDHC-treated cells as expected (see Fig. 1A).

To understand the role of the dynein motor and late endosomes in HIV-1 replication, we next analyzed the subcellular localization of vRNA, Gag, and LAMP-1 in HIV-1-expressing cells using a combined FISH and IF analysis. In control conditions (siNS), vRNA was distributed throughout the cytoplasm in a punctate pattern, at the plasma membrane, and partially co-localized with both Gag and LAMP-1 in the perinuclear region (Fig. 2A, top row). The proportion of vRNA that co-localized with Gag and, conversely, the proportion of Gag that co-localized with vRNA reached 37% (±7, S.D.) and 40% (±8, S.D.), respectively.

When dynein motor function was disrupted by the depletion of DHC by siRNA or by p50/dynamitin-HA expression, LAMP-1 localized to the cell periphery, and a proportion of both vRNA and Gag redistributed with LAMP-1 (Fig. 2A, rows 2 and 3). RILP-Myc overexpression induced an enrichment of vRNA and Gag at the MTOC, along with a separate population of Gag in the periphery (Fig. 2A, row 4). This was found in 100% (n = 28 cells), 98% (n = 44 cells), and 95% (n = 41 cells) siDHC-, p50/dynamitin-HA-, and RILP-Myc-treated cells, respectively, in the experiment shown in Fig. 2A. The results were confirmed by acquiring multiple 400 nm two-dimensional focal planes through cells demonstrating that the co-localization between

**FIGURE 2.** vRNA and Gag co-localize with late endosomes in a dynein-dependent manner. HeLa cells were transfected with HIV-1 proviral HxBRU DNA with siNS, siDHC, p50/dynamitin-HA, or RILP-Myc and fixed for IF/FISH analysis. A, vRNA (green), LAMP-1 (red), and Gag (blue). Arrows show accumulations of vRNA, Gag, or LAMP-1 in cells. B, results from co-localization analyses are shown using the Manders’ co-localization coefficient as described under “Experimental Procedures.” Error bars represent standard deviations of the mean from three experiments. Scale bar, 10 μm.
LAMP-1 and vRNA or Gag was not dependent on the laser focal plane scanned (supplemental Fig. S1). vRNA and Gag were also found tightly associated to late endosomal membranes even when the N-terminal deletion mutant of RILP (RILP/H9004N) was expressed, a mutant that fails to recruit dynein to late endosomes leading to the dispersal of late endosomes in cells (supplemental Fig. S2) (51). Importantly, our analyses revealed that the co-localization of LAMP-1 with either vRNA or Gag modestly increased by 50–70% when late endosomes were peripherally localized in siDHC- and p50/dynamitin-HA-treated cells (Fig. 2B). Therefore, there is a direct relationship between peripherally localized vRNA- and Gag-containing LAMP-1+/membranes and virus production underscoring the importance of this dynein-dependent and regulatable population of endosomes during viral expression.

A time course experiment was then performed to determine when we could first detect the coordinated changes in the localization of vRNA, Gag, and LAMP-1+ late endosomes. In this experiment, cells were transfected and then fixed at 12, 24, and 48 h post-transfection. p50/dynamitin-HA and RILP-Myc overexpression influenced the localization of vRNA and Gag in the manner as previously observed as early as 12 h post-transfection (Fig. 3). At this time point, vRNA was observed in the nuclei in several transfected cells. Later, vRNA was predominantly cytoplasmic along with higher signal intensities for both Gag and vRNA. The distribution of Gag and vRNA was the same for all time points. For example, p50/dynamitin-HA caused Gag and vRNA to relocalize to the periphery. This phenotype was found in >80% of cells at both 24 and 48 h (n = 24 cells at each time point in the experiment shown). RILP-Myc promoted the strong localization of vRNA along with LAMP-1 membranes at the MTOC. At 12 h for instance, vRNA and LAMP-1 were found at the MTOC in ~30% of HIV-1-expressing cells (n = 18 cells in the experiment shown), but this localization pattern became more evident at 24 and 48 h, and was found in >90% of cells (n = 20 cells in the experiment shown). This likely corresponds to the expression levels of RILP-Myc in the cells. The steady-state vRNA signal intensity in the cytoplasm decreased with time in RILP-Myc-expressing cells, indicating that the vRNA was rapidly rerouted to the MTOC. Results from these experiments indicate that vRNA localization depends on dynein motor function and that vRNA association with LAMP-1+ endosomal membranes occurs during both the early and later phases of HIV-1 gene expression.

When cells were fractionated into cytoplasmic, membrane, and nuclear compartments, we could not detect any changes in the subcellular compartmentalization of Gag (supplemental Fig. S3). Furthermore, the membrane association of vRNA (~10% of the total) was not altered by RILP-Myc expression (supplemental Fig. S4), nor was it modulated when vRNA redistributes to the MTOC upon heterogeneous nuclear ribonucleoprotein A2/B1 depletion in cells3 (data not shown) (17), indicating that the change to vRNA localization does not necessarily correspond to changes in its association to membranes. Thus, the marked recruitment of vRNA to LAMP-1+ membranes is likely to be achieved via another means (see below).

Gag Myristoylation Is Important for the Co-trafficking of vRNA and Gag on LAMP-1+ Membranes—The association of HIV-1 Gag to membranes is primarily achieved by the myristoylation of its N-terminal matrix domain with additional contributions from basic amino acids in this domain. vRNA is

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selected by Gag via the interaction that occurs between the NC domain of Gag and the psi RNA packaging signal in the 5' untranslated region of the vRNA. This event occurs near the MTOC in cells (41) and leads to the eventual encapsidation of vRNA into progeny virus particles at virus assembly sites. Thus, Gag could potentially link vRNA to membrane-trafficking pathways. To examine this notion, the HIV-1 provirus [SVBH10(myr +)] and an isogenic provirus harboring a mutated myristoylation signal [SVBH10(myr -)] were tested in our system. As expected, the processing of Gag and Gag/Pol was found to be aberrant in SVBH10(myr -)-expressing cells, because there was an accumulation of Gag/Pol (pr160 Gag/Pol) and the Gag processing intermediate, p25 (Fig. 4A). Some of these defects have been explained by an impaired capacity of the mutant Gag protein to bind membranes (69).

In SVBH10(myr +)-expressing cells, the overexpression of p50/dynamitin-HA or of RILP-Myc resulted in the expected peripheral or MTOC localization of late endosomes, Gag, and vRNA. In SVBH10 (myr -)-expressing cells, however, LAMP-1, Gag, and vRNA had distinct localization patterns and neither viral component co-localized with LAMP-1 to any appreciable extent. In untreated SVBH10(myr -) cells, both Gag and vRNA were diffuse throughout the cytoplasm, and their localization patterns were not changed when late endosomes were relocalized under p50/dynamitin-HA and RILP-Myc overexpression conditions. This was particularly evident in RILP-Myc-expressing cells where little, if any, vRNA co-localized with LAMP-1 membranes at the MTOC (Fig. 4B). Generally, the co-localization between vRNA, Gag, and LAMP-1 was 5–10% higher when SVBH10(myr +) was expressed compared with that found for other HIV-1 proviruses used in this work. We calculated that, in HIV-1-expressing cells, 44% (±3 S.D.) of Gag co-localized with LAMP-1+ membranes at the MTOC (Fig. 4C). A similar observation was found for vRNA such that 52% (±2 S.D.) of vRNA co-localized with LAMP-1+ membranes (Fig. 4C). A dramatic loss of the association between both Gag or vRNA and LAMP-1 was observed during the expression of SVBH10(myr -) (Fig. 4C). The same trend in co-localization could be found in SVBH10(myr -)-expressing cells transfected with either p50/dynamitin-HA or RILP-Myc (data not shown). These results were confirmed by performing confocal imaging of multiple focal planes through the cells. This
analysis revealed that there was a coordination of vRNA, Gag, and LAMP-1 + membranes in myr + conditions, whereas this was not the case in myr − conditions (supplemental Fig. S5). Thus, Gag myristoylation is important for vRNA localization to late endosomal membranes. Myristoylation of Gag is also needed for vRNA localization and may provide a functional link between Gag/vRNA and cellular membrane tethering and transport.

Mutations in the NC domain of Gag can have severe defects on genomic RNA dimerization, packaging, infectivity, and reverse transcription (Refs. 53, 54; reviewed in Ref. 70). This region of Gag also influences Gag multimerization and the association of Gag with membranes in studies that employed HIV-1 proviral mutants harboring point mutations, N-terminal and zinc finger deletions of NC. The expression of some of these mutants was shown to lead to altered cytoplasmic staining of Gag and vRNA (29, 71, 72) as well as to a diminished interaction between Gag and vRNA (29). These results point to a general model predicting that NC mutations modulate steady-state Gag membrane association and assembly. To further demonstrate the importance of Gag membrane association on the interaction between vRNA and LAMP-1 membranes, we tested two HIV-1 proviral NC mutants. The first harbors a deleted N-terminal zinc finger #1 (NC/Delta1) (53) and the second harbors point mutations in the linker domain between the two zinc fingers of NC (NC/S3E (54)) (Fig. 5A). When we assessed the localization of vRNA in relation to LAMP-1 by confocal microscopy, vRNA was found to co-localize very well with LAMP-1 + membranes, to a similar extent that we reported above (Fig. 5B). Strikingly, however, we did not detect any appreciable overlap between the vRNA and LAMP-1 signals in cells when the NC mutants were expressed (Fig. 5B). We also found identical results using a NC mutant that lacked the zinc finger #1 but harbored two C-terminal zinc fingers #2, and furthermore, vRNA did not redistribute to the MTOC when RILP-Myc was expressed in cells expressing these NC mutants.4 When we assessed Gag localization in NC/WT conditions, it co-localized with LAMP-1 + membranes to the same extent that we observed earlier, indicated by the resulting magenta staining (resulting from red and blue fluorescence signals) in these cells (Fig. 5C). However, the localization of Gag was found to be distinct from that of LAMP-1 when the NC/Delta1 and NC/S3E mutants were expressed (Fig. 5C), and this was reflected in a loss in co-localization between LAMP-1 and vRNA or Gag when we calculated the Manders’ overlap coefficients.4 The phenotypes shown were found in 90–95% of cells in each of three independent experiments, examining 50–100 HIV-1-expressing cells per condition per experiment. Overall, the HIV-1 NC assembly mutants disengage both the vRNA and Gag from LAMP-1 membranes and corroborate our findings obtained with SVBH10 (myr −).

vRNA Localization to Late Endosomes Does Not Result from Endocytosis of Virions—HIV-1 assembly occurs in a cell-type-dependent manner at either the plasma membrane or on internal late endosomal membranes (37). Recent reports using transf dominant negative proteins to block endocytosis indicate that the accumulation of HIV-1 Gag protein on endosomal vesicles results from endocytosis of viruses or membrane-associated Gag derived from the plasma membrane and does not reflect productive viral replication (73). We therefore evaluated the distribution of vRNA in conditions when endocytosis was blocked. To test if vRNA localization to late endosomal membranes resulted from endocytosis of virions or vRNA, we expressed trans dominant-negative mutants (DN) of Eps15 and Rab5 in cells expressing HIV-1. Eps15 DN/GFP inhibits clathrin recruitment and endocytosis, and Rab5 DN/GFP blocks transfer of endocytic cargo to early endosomes (58). Both DN mutant proteins are fusion proteins with GFP and are readily detectable in cells by fluorescence microscopy. In preliminary experiments, we evaluated the ability of the DN/GFP mutants to block the uptake of extracellular Texas Red-coupled transferrin (Trf). In cells that expressed either of the DN/GFP mutants, substantially reduced clathrin Trf uptake was observed (77% of cells expressing Rab5 DN/GFP (n = 22 cells) and 94% of cells expressing Eps15 DN/GFP (n = 17 cells); data not shown). In HIV-1-expressing cells, abundant signals for the vRNA were identified by FISH in cells in which Trf uptake was blocked by both DN proteins demonstrating that the clathrin staining for vRNA was not due to endocytosis events (Fig. 6A). Cellular Gag expression levels were unchanged when Eps15 DN/GFP and Rab5 DN/GFP proteins were expressed as determined by Western blotting (Fig. 6B). We then stained cells for LAMP-1 by IF and vRNA by FISH and found that the localization of vRNA with LAMP-1 was not affected in cells expressing Eps15 DN/GFP or Rab5 DN/GFP, because vRNA remained strongly co-localized with LAMP-1 in both cytoplasmic and juxtanuclear positions (Fig. 6, C and D). Therefore, vRNA localization with late endosomes is likely not the result of an accumulation of endocytosed viruses or vRNA but rather represent trafficking intermediates for HIV-1 vRNA during the late viral replication stages.

Recent work has shown that the activity of CD317 (also known as tetherin) is inhibited by the HIV-1 auxiliary protein Vpu, and this promotes the release rather than the retention and internalization of de novo synthesized virus particles at the plasma membrane (74). The HxBRU proviral construct that we used in most of the preceding experiments does not encode Vpu. We therefore attempted to rule out any effects of virus internalization in our FISH and IF studies. HeLa cells were transfected with a pNL4.3 or a Vpu-negative pNL4.3 provirus (pNL4-3/Vpu −), and the redistribution of vRNA was stimulated with either p50/dynamitin-HA or RILP-Myc. vRNA showed a punctate staining pattern and a marked co-localization with LAMP-1 membranes in pNL4.3-transfected cells (Fig. 7). In these cells, p50/dynamitin-HA caused a recruitment of a proportion of vRNA and LAMP-1 to the periphery and RILP-Myc caused the majority of vRNA to localize to MTOCs, as observed above. The same results were obtained with pNL4.3/ Vpu − (Fig. 7). These results indicate that Vpu along with active tetherin do not affect the vRNA localization patterns that we observed.

4 M. P. Milev, M. Lehmann, and A. J. Mouland, data not shown.
FIGURE 5. Gag nucleocapsid mutations lead to a loss of co-localization between LAMP-1 and vRNA or Gag. HeLa cells were transfected with wild-type pNL4.3, or pNL4.3 DNA harboring either a deletion of the N-terminal zinc finger #1 (NC/Delta1) or a zinc finger linker domain mutant, NC/S3E as indicated (A). Cells were fixed at 30-h post-transfection and processed for IF/FISH co-analyses. vRNA and LAMP-1 are presented in black and white with a merged color image in B. Localization patterns for Gag (blue) and LAMP-1 (red) are presented in merged images in C. Scale bar, 10 μm.
To further examine the influence of endocytosis on vRNA localization, we determined how vRNA would appear by FISH analysis when HIV-1 is programmed to infect cells via endocytosis. HeLa cells were either incubated with purified and infectious wild-type HIV-1 (HIV-1\textsuperscript{env}), or they were infected with VSV-G-pseudotyped HIV-1 (HIV-1\textsuperscript{VSV-G}), a virus that will gain entry into cells by endocytosis (65). The apparent infection frequencies of HeLa cells were negligible for HIV-1\textsuperscript{env} (0.01–0.001% of cells) as expected and likely to be due to background GFP signals. For pseudotyped HIV-1\textsuperscript{VSV-G} an infection rate of 20–25% was achieved (Fig. 8A). Incubation of HeLa cells with HIV-1\textsuperscript{env} resulted in small, punctate staining for vRNA (Fig. 8B) and in ~5% of cells, larger intracellular accumulations of dispersed and faint vRNA signals at later time points (data not shown). In pseudotyped HIV-1\textsuperscript{VSV-G} infections, we identified larger intracellular punctae of vRNA in ~10% of cells (n = 120 cells) that likely represented endocytosis of virus particles (Fig. 8B). The vRNA staining coincided with LAMP-1 but, again, the signals were very different in number, shape, size, location, and signal intensity compared with the vRNA staining results in our transfection studies. Thus, endocytosed HIV-1 virions are readily differentiated from larger and robust signals for vRNA and Gag in HIV-1-producing cells following transfection.

Because we have ruled out that the vRNA and Gag signals in HIV-1-producing cells are likely not due to endocytosed virus particles, we performed ultrastructural analyses to determine if intracellular virus particles or intracellular budding structures could be detected. Cells were transfected with proviral DNA (HxBRU) and siNS, siDHC, p50/dynamitin-HA, or RILP-Myc and processed for electron microscopy analysis. In the control siNS-treated cells, virus particles were found predominantly to bud at the cell surface. Multivesicular bodies containing intraluminal vesicles could be detected that had sizes smaller than what would be expected for virions (<100 nm, Fig. 9A). In ~4% of cells examined (examining 30–50 cells per condition per experiment), internalized virus-like structures in large vesicular structures were found (not shown) (75). In DHC-depleted cells that were identified by the presence of fragmented Golgi membranes, abundant virus particles budded from the surface (Fig. 9B). In p50/dynamitin-HA-overexpressing cells, which showed disrupted Golgi stacks and vesicular structures at the cell periphery (Fig. 9C), abundant numbers of virions were found at the cell surface, consistent with the increase in virus production in this condition (Fig. 1B). RILP-Myc expression causes the clustering of late endosomes and multivesicular bodies at the MTOC (50, 51), but virus particles were not detected at this juxtanuclear domain (Fig. 9D). Overall, virus structures were abundant at the cell surface, but there was little evidence for intracellular virus particles (internalized or budding on internal membranes) under the different conditions used in this study. Thus, it is likely that vRNA and Gag are trafficked on late endosomal membranes/vesicles, and this could be achieved in the context of a viral ribonucleoprotein complex that is tethered to
endosomal membranes via Gag. Consistently, the RNA trafficking protein, Staufen1, co-traffics with these same populations (supplemental Fig. S6).

**DISCUSSION**

Our experiments demonstrate that there is an intimate relationship between the trafficking of endosomal membranes, Gag, and vRNA as evidenced by the high degree of co-localization with LAMP-1+ membranes in cytoplasmic and juxtanuclear loci. We also show that the localization of these viral and cellular components is coordinated by a similar mechanism in experiments in which we disrupt dynein motor function or promote its recruitment to the MTOC. Our data identify the importance of dynein motor function and LAMP-1+ membranes on virus production (Figs. 1–3). The changes in vRNA localization were dependent on the myristoylation and on an intact NC domain of Gag (Figs. 4 and 5) consistent with earlier biochemical results indicating that the NC domain plays a role in the association of vRNA with late endosomal membranes (29). The co-localization of LAMP-1 and vRNA is almost completely abrogated by mutations in the Gag NC region. However, we cannot rule out contributions that these mutations would have on preventing the binding of Gag and vRNA that would further contribute to the uncoupling of the vRNA from LAMP-1+ membranes (Fig. 5). These data show that membrane association of Gag is required for Gag/vRNA trafficking. The ability to alter vRNA localization indicates that vRNA trafficking is a regulatable and active process that can be disrupted with treatments that block directed and energy-dependent intracellular traffic.

What could be the function of the dynein motor in the transport of HIV-1 vRNA? Dynein and kinesin motors share many features. For example, both reside in the same RNA-trafficking ribonucleoprotein complex (19, 76) and on late endosomes (77). In addition, dynein and kinesin are found on inbound and outbound complexes (78), and they have the same binding domains on microtubules (79). Nevertheless, they do have opposing motor activities: dynein promotes minus-end transport of cargo toward the MTOC, and most kinesins promote plus-end transport toward the cell periphery on microtubules. One proposal on how directionality of intracellular traffic is determined involves a simple tug-of-war between these opposing motor activities (76, 78). Alternatively, an “on-off” mechanism could be mediated by a molecular switch (80). Thus, based on our results we propose that, following transcription and nuclear export, the vRNA transits toward the MTOC (11, 17). Gag synthesis, which is not affected by blocking dynein function at steady-state, would then allow for some of the first interactions with vRNA, consistent with biophysical and imaging data that demonstrate that the first Gag-vRNA contacts nucleate at this site (17, 41). This also represents a site through which Gag transits as documented in live cell assays (39, 81).

Although the co-opting of dynein motor function following HIV-1 virus entry likely depends on interactions with mature
virion-associated Gag proteins (17, 82), we propose that during viral egress a switch from dynein to kinesin motor activity could occur by high levels of de novo synthesized Gag at this stage of viral replication. This would first prevent Gag (and vRNA)-containing late endosomal membrane or vesicle positioning at a juxtanuclear location, and instead favor targeting of late endosomes to the periphery, an event that would enhance virus production.

Late endosomal targeting of vRNA to the plasma membrane could be accomplished by a variety of mechanisms. This could include post-translational modifications to modulate kinesin/dynein motor function to allow for rapid changes in directionality (78). For HIV-1, viral egress of vRNA and Gag could be achieved by late endosome to Golgi trafficking, which is mediated by Rab9 (31), with a subsequent trafficking step to the plasma membrane. DG, dispersed Golgi and vesicles; GS, Golgi stacks and vesicles; EV, mature or invaginating endocytic vesicle; MVB, multivesicular body; and MIT, mitochondrion. Scale bars (500 nm) are indicated in each panel.

The notion that RNAs are trafficked and require the coordinated movement of RNPs alone or in the context of membranes is supported by our experiments and suggested by others (28, 87). Consistently, the localization of components of the HIV-1 ribonucleoprotein, including StauIen1, is modulated by treatments that reroute dynein-dependent late endosomal vesicles (supplemental Fig. S6). There may even be factors, such as StauIen1, that can regulate vRNA-dependent Gag multimerization to positively regulate Gag assembly on membranes (33), as well as vRNA fate in the cytoplasm during HIV-1 replication (49). The loss of co-localization between LAMP-1 and vRNA or Gag that was found in the experiments using the myristoylation and NC mutants (Figs. 4 and 5 and supplemental Fig. S5) is consistent with observations that membrane association of Gag is required for assembly (88). In addition, the changes in vRNA localization when we expressed p50/dynamitin-HA or RILP-Myc or treated cells with siDHC were not due to de novo synthesized or internalized virus particles (Figs. 6–9) (73, 74, 89). Therefore, vRNA is likely trafficked in the context of a ribonucleoprotein in association to LAMP-1 membranes. Indeed, we have previously shown that vRNA exists in cytoplasmic ribonucleoproteins containing Gag and Staufen1 (46) and that HIV-1 RNAs are directionally trafficked in large cytoplasmic ribonucleoproteins (21) supporting the data obtained here in HIV-1-expressing cells. Our membrane flotation assays also support this notion in that the compartmentalization of vRNA does not change in RILP-Myc-expressing cells despite the striking subcellular redistribution of vRNA (supplemental Fig. S4). The HIV-1 ribonucleoprotein (containing Gag, vRNA, and the cellular proteins Staufen1 and UPF1) (59) is nevertheless dynamic, because Gag can be separated from it when cells are stressed with puromycin.5

Endosomal sorting complex required for transport proteins are not only essential for HIV-1 assembly but also play a role in mRNA transport in Drosophila involving a Staufen ribonucleoprotein (90). The association between endosomal sorting complexes for transport proteins and RILP (91, 92) supports the idea that late endosomal membranes positively promote vRNA traffic. RILP is also involved in the control of late endosomal transport by directly interacting with the small GTPase Rab7 and the dynnein/dynactin component, p150glued (93). This biochemical evidence suggests that functional relationships could exist between endocytic membranes, molecular motors, vRNA trafficking, and HIV-1 assembly.

Our results provide new information on how the HIV-1 vRNA is trafficked before being assembled into virions. We demonstrate that at least two populations of both Gag and vRNA exist in cells: one that is displaced along with LAMP-1 membranes when dynein is disrupted and one (or others) that is not, which is consistent with earlier observations for Gag (reviewed in Ref. 94). The population containing Gag,

5 L. Abrahanyan and A. J. Mouland, unpublished observation.
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vRNA, LAMP-1, and Staufen1 is displaceable by the disruption of dynein motor activity. Our data indicate that this population of vRNA is packaged into progeny virions during assembly, because the changes in the cellular distribution of Gag and vRNA lead to coordinated changes in virus production but had little impact on viral infectivity (in siDH and p50/dynamitin conditions). The co-trafficking of Staufen1 with Gag and vRNA provides additional support for the importance of these populations in HIV-1 assembly (46, 47, 49, 59). Our results suggest that dynein motor activity may be a suitable target for intervention, not only during ingress (82, 95) but also for the regulation of vRNA, Gag, and membrane traffic during viral egress and assembly.

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