Feline Immunodeficiency Virus Gag Is a Nuclear Shuttling Protein

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Lentiviral genomic RNAs are encapsidated by the viral Gag protein during virion assembly. The intracellular location of the initial Gag-RNA interaction is unknown. We previously observed feline immunodeficiency virus (FIV) Gag accumulating at the nuclear envelope during live-cell imaging, which suggested that trafficking of human immunodeficiency virus type 1 (HIV-1) and FIV Gag may differ. Here we analyzed the nucleocytoplasmic transport properties of both Gag proteins. We discovered that inhibition of the CRM1 nuclear export pathway with leptomycin B causes FIV Gag but not HIV-1 Gag to accumulate in the nucleus. Virtually all FIV Gag rapidly became intranuclear when the CRM1 export pathway was blocked, implying that most if not all FIV Gag normally undergoes nuclear cycling. In FIV-infected feline cells, some intranuclear Gag was detected in the steady state without leptomycin B treatment. When expressed individually, the FIV matrix (MA), capsid (CA), and nucleocapsid-p2 (NC-p2) domains were not capable of mediating leptomycin B-sensitive nuclear export of a fluorescent protein. In contrast, CA-NC-p2 did mediate nuclear export, with MA being dispensable. We conclude that HIV-1 and FIV Gag differ strikingly in a key intracellular trafficking property. FIV Gag is a nuclear shuttling protein that utilizes the CRM1 nuclear export pathway, while HIV-1 Gag is excluded from the nucleus. These findings expand the spectrum of lentiviral Gag behaviors and raise the possibility that FIV genome encapsidation may initiate in the nucleus.

Gag is both necessary and sufficient for retrovirus particle formation and budding. The protein is translated on free polysomes and targeted to the plasma membrane, where virus particles are assembled (16). A central question is whether Gag and the viral genomic RNA (gRNA) associate at the plasma membrane or earlier during assembly and, if so, whether this occurs in the cytosol; in association with organelles, e.g., cotranslationally; or even in the nucleus, as was described previously for an alpharetrovirus (17). Human immunodeficiency virus type 1 (HIV-1) gRNAs become anchored at the plasma membrane before particle assembly is detectable, but it is not clear whether the Gag-gRNA complexes of this and other lentiviruses first form in the cytoplasm and then transit to the plasma membrane or the gRNA traffics there independently (26, 27). Biochemical experiments supported the former scenario, with a monomer or low-order multimers forming on HIV-1 gRNAs and higher-order multimer formation depending on subsequent plasma membrane interactions (33).

Gag is encoded by the full-length unspliced viral RNA. To circumvent the cellular checkpoint that prevents the export of intron-containing mRNA, lentiviruses express Rev, which functions as an adaptor between the cellular karyopherin CRM1/exportin-1 and a Rev response element (RRE) located in the 3’ region of unspliced viral RNAs (9). However, the main cellular function of the CRM1 nuclear export pathway is the export of cellular proteins containing a nuclear export signal (NES) as well as ribosomal subunits, 5S ribosomal RNAs and uridylicate-rich small nuclear RNAs (UsnRNAs) (14). In contrast, most cellular mRNAs are exported from the nucleus via NXF1/NXT. This pathway is exploited by the simple orthoretrovirus Mason-Pfizer monkey virus (MMPV), which has a constitutive RNA transport element (CTE) that recruits NXF1 directly (4, 19).

The trafficking itinerary and assembly competence of HIV-1 Gag can be influenced by the nuclear export history of the mRNA from which it is translated (1, 25, 52). If the HIV-1 RRE is replaced by the MPMV CTE, the normally Rev-dependent unspliced RNA exits the nucleus efficiently, but Gag translation is inefficient (7). Replacement of the RRE by the hepatitis B virus posttranscriptional regulatory element (PRE) results in normal Gag expression but reduces the budding efficiency by 10-fold (24). Cell- and species-specific effects have also been observed. For example, Gag expressed from a Rev-dependent RNA is assembly incompetent in some rodent cell lines, but replacing of the RRE with the CTE restores assembly (52).

We recently used protein and RNA labeling in live cells to show that feline immunodeficiency virus (FIV) Gag and gRNA accumulate, independently of each other, at the nuclear envelope (30). Discrete foci of colocalized FIV Gag and gRNA were also observed at the cytoplasmic surface of the nuclear envelope. In contrast, and consistent with observations reported previously by many laboratories, we never saw HIV-1 Gag colocalize with the nuclear envelope. While HIV-1 gRNA was detectable there, this was observed much less frequently than with FIV gRNA, and it was not seen in most cells (30). For both lentiviruses, gRNA was visualized at the plasma membrane only if Gag was coexpressed and the gRNA packaging signal was intact. Here we pursued these observations further by determining whether either lentiviral Gag protein accesses the intranuclear compartment. The results reveal a surprising, sharp difference between the two lentiviruses.

MATERIALS AND METHODS

Plasmids, cells, transfections. Cyan fluorescent protein (CFP) refers to the enhanced version (eCFP), pFIVGag-CFP (also termed pFP93gagCFP) and pHIVGag-CFP were described previously (30). pFIVGag-CFP is derived from the FIV packaging plasmid pFP93 (44) (a diagram of pFP93 is available upon request).
using pFIVGag-CFP as a template. pFP93Prm was constructed by overlap extension PCR that introduced an Asp-to-Gly change in the LLDTG motif (35) in the protease active site of pFP93 (the D30 GAC codon was changed to GAG). The deletion of pol from pFP93 (to yield pFP93ΔPol) was achieved with an EcoRI-XcaI adaptor that was ligated between the EcoRI site near the gag C terminus and an XcaI site located at nucleotide (nt) 232 of the integrase gene. This eliminated all of pol except for the 614-nt integrase gene 3’ remnant; therefore, Gag but no pol-encoded viral enzymes were expressed. Correct plasmid constructions were all confirmed by sequencing.

pCT5 (cytomegalovirus [CMV] promoter fused to the FIV R repeat at TATA box plasmid 3) is used to produce infectious, replication-competent FIV in human cells, which is enabled because the human cell-inactive 5’ FIV U3 element was replaced with the human CMV (hCMV) promoter just upstream of the 5’ R’ repeat (41, 42). pCT5 was derived from the infectious 34TF10 molecular clone previously isolated from a lambda phage library by Talbott et al. (53). Note that pCT5 encodes the wild-type (WT), normally infectious 34TF10 virus, since it expresses the full 34TF10 R-to-R proviral transcript with a normal 5’ cap site and a normal 3’ U3 (41, 42). pCT5neo is identical to pCT5 except for a 31-nt insertion that causes an envelope frameshift in the central region of env; pCT5neo was used to produce vesicular stomatitis virus glycoprotein G (VSV-G)-based, single-cycle, full-length FIV (29).

CMVΔΔ88.9 expresses HIV-1 Gag-Pol, Tat, and Rev (56). NL4-3-ΔΔ426, also referred to here and in our previous papers as HIV-1luc,i sa, is a single-cycle HIV-1 luciferase reporter virus (36); its provirus differs from HIV-1 NL4-3 in only three ways: it has a 426-nt deletion in env, a frameshift in vpr, and a replacement of nef with the firefly luciferase gene (luc). Pseudotyped Δenvelope FIV 34TF10 (expressed by pCT5neo) and HIV-1luc,i sa were produced in 293T cells as previously described (44).

Crandell feline kidney (CrFK) (8), 293T, HeLa, and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. DNA transfection was carried out with calcium phosphate coprecipitation (293T, HeLa, and CrFK cells) or Fugene 6 (Roche Applied Science) (COS7 cells). Titers of FIV were determined by the quantification of infectious foci at 48 h in CrFK cells using the immunofluorescence method described below.

Indirect immunofluorescence, live-cell imaging, and immunoblotting. HeLa cells were transfected with 2 μg of plasmid or transduced with VSV-G-pseudotyped CT5neo or VSV-G-pseudotyped HIV-1luc,i sa Cells were either left untreated or treated with leptomycin B (LMB; Sigma-Aldrich) at 10 ng/ml (18 nM) for 3 h. A CRM1-dependent green fluorescent protein (GFP) shuttle indicator, Rev1.4-GFP-NES, containing the NES from adenomatous polyposis coli tumor suppressor protein, was used as a positive control for the LMB blockade of the CRM1 pathway (23). For indirect immunofluorescence, cells were fixed with 3% paraformaldehyde for 15 min on ice, washed with phosphate-buffered saline (PBS), then permeabilized with 0.2% Triton X-100 for 10 min at room temperature, washed, and blocked with 3% low-fat milk for 30 min. Cells were then incubated with monoclonal antibody (MAb) PAB2-11 (1:200 dilution; Amer sham) or with monoclonal antibody (MAb) PAK3-2C1 (1:200; Custom Monoclonal Antibodies International, Sacramento, CA), which recognizes FIV capsid, or with monoclonal antibody 183-H12-5C to HIV-1 p24, from the NIH AIDS Reference and Reagent Program, at a 1:4,000 dilution overnight at room temperature in a humidified chamber. For fibrillarin, a primary rabbit antibody (Novus Biologicals) was used at a 1:200 dilution. After cells were washed with PBS, they were blocked again and then incubated with an anti-mouse Alexa Fluor 594- or anti-rabbit Alexa Fluor 488-conjugated secondary antibody (1:1,000; Molecular Probes) for 30 min, washed, and mounted in Prolong Gold (Molecular Probes). Slides were analyzed on a Zeiss LSM 510 confocal laser scanning microscope equipped with an Axiovert 200 M inverted platform using a Plan-Apo 488-conjugated secondary antibody (1:1,000; Molecular Probes) for 30 min, washed, and mounted in Prolong Gold (Molecular Probes). Slides were analyzed on a Zeiss LSM 510 confocal laser scanning microscope equipped with an Axiovert 200 M inverted platform using a Plan-Apochromat 100× (1.4-numerical-aperture) oil immersion objective. For live-cell imaging, HeLa cells or COS7 cells were seeded into Lab-Tek glass-bottom chamber slides (Nunc) and observed at 20 h posttransfection with the above-described Zeiss LSM 510 confocal laser scanning microscope. CFP was excited with an argon laser at 458 nm, and emission was detected with a band-pass filter of 475 to 525 nm. The quantification of the nuclear translocation of FIV Gag-CFP in HeLa and COS7 cells was done with Zeiss
KS400 image analysis software. The mean fluorescence intensity (MFI) was measured in the nucleus or the cytoplasm, and the area of the two compartments was determined. The total MFI was determined by multiplying the MFI with the area: % = total MFI (nucleus)/total MFI (nucleus + cytoplasm).

For immunoblotting, cells were lysed in 1× radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) containing protease inhibitors (Complete Mini; Roche). Fifteen micrograms of cell lysate was separated on a reducing 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membranes were blocked in 0.2% l-block (Tropix)–0.1% Tween and then probed with a monoclonal anti-GFP/CFP antibody (1:5,000; BD Biosciences), referred to below as the anti-CFP antibody. As a secondary antibody, a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:1,000; Calbiochem) was used. Bands were visualized with Lumi-Light detection reagent (Roche).

**RESULTS**

**Nuclear import and CRM1-dependent export of FIV Gag-CFP.**

To study subcellular localization in living cells, we fused CFP to the FIV Gag C terminus (pFIVGag-CFP) (Fig. 1A). As we previously observed (30), in HeLa or COS7 cells, FIV Gag-CFP has a mostly dispersed but nonhomogeneous cytoplasmic distribution (Fig. 1B and D). However, when cells were incubated for 3 h with leptomycin B (LMB), a specific inhibitor of CRM1-mediated nuclear export (14), Gag-CFP accumulated in the nucleus (Fig. 1C and E). In contrast, CFP was distributed as expected between the nucleus and cytoplasm and was unaffected by LMB (Fig. 1F and G). The nuclear accumulation of Gag-CFP was partial, with an apparent majority of the protein remaining in the cytoplasm. In both cell types, Gag-CFP also localized in nucleoli, while CFP did not (Fig. 1C, E, and K).

To quantify LMB-induced nuclear translocation, the mean fluorescence intensity (MFI) in the nucleus and the cytoplasm was measured, and the area of the compartments was determined. The total MFI was calculated (MFI × area) for the nucleus and the cytoplasm, and the ratio of the total MFI of the nucleus to the total MFI of the cell (nucleus plus cytoplasm) was determined (Fig. 1H). In untreated HeLa cells, 6.2% of the total fluorescence was nuclear. In LMB-treated cells, however, the nuclear proportion increased 6-fold, to 37.7%. In COS7 cells, the fluorescence intensity increased 2.7-fold, from 7.8% for untreated cells to 21.2% with LMB treatment. To exclude the possibility that this nuclear localization represented the import of an LMB-induced proteolytic cleavage fragment of Gag-CFP, Western blots of HeLa cell lysates were probed with an antibody to CFP. As demonstrated in Fig. 1I, FIV Gag-CFP remained intact in the presence and absence of LMB, with neither free CFP nor any other CFP-containing fragment being generated. Therefore, the nuclear fluorescence observed in Fig. 1C and E is due to the nuclear trafficking of full-length Gag-CFP, and the apparent restriction to the cytoplasm in the steady state in untreated cells requires CRM1-mediated export. We next imaged, in a similar fashion, FIV Gag fused to a strictly monomeric fluorescent protein, mCherry (Fig. 1I). The use of mCherry resulted in an increased nuclear versus cytoplasmic localization in the presence of LMB (Fig. 1I). Nucleolar concentration was also evident in some cells, although this was less frequent than with CFP as the fusion partner (<20% of cells). z stacks of FIV Gag-CFP-expressing cells confirmed a lack of nuclear localization in the steady state and abundant nuclear accumulation with a nucleolar concentration after LMB treatment (Fig. 1K). We readdress nucleolar localization below.

**HIV-1 Gag-CFP does not traffic to the nucleus.** To establish a comparative benchmark for these CRM1 export pathway-dependent effects, we analyzed the subcellular localization of HIV-1 Gag-CFP in parallel (Fig. 2). HIV-1 Gag-CFP exhibited a cytoplasmic localization with punctate foci as well as a diffuse signal in both cell types and strong plasma membrane accumulation in COS7 cells. Similarly to FIV Gag-CFP, HIV-1 Gag-CFP was excluded from nuclei. However, in clear contrast to FIV Gag-CFP, LMB treatment did not result in nuclear HIV-1 Gag-CFP accumulation. No difference in the subcellular distributions of HIV-1 Gag-CFP was observed for LMB-treated versus untreated cells.

**Nuclear import and retention of unlabeled FIV Gag but not HIV-1 Gag.** In order to exclude the possibility that the CFP or mCherry moiety causes an aberrant nuclear translocation of FIV Gag, we determined the trafficking of the unmodified Gag protein of HIV-1 and FIV in the presence and absence of LMB. In addition, we employed both virus infection and DNA transfection in these experiments (Fig. 3).

Cells were first transfected with standard FIV and HIV-1 vector packaging plasmids that produce Gag and Gag-Pol polyproteins from Rev-dependent RNAs. Indirect immunofluorescence was performed with antibodies to FIV capsid or HIV-1 capsid. Again, as seen with FIV Gag-CFP, a pronounced nuclear retention of HIV Gag was observed upon LMB treatment (Fig. 3A and B). When the amounts of nuclear accumulation were compared, even more native FIV Gag than FIV Gag-CFP was nuclear (compare Fig. 1C, E, J, and K with 3B). The nucleolar concentration, although consistently detectable, was much less common than with the Gag-CFP fusion, varying from 1 to 10% of cells in multiple experiments. In
clear contrast to FIV Gag, LMB did not result in a nuclear translocation of unlabeled HIV-1 Gag (Fig. 3E and F).

We then carried out viral infection rather than transfection to confirm these results and verify that they occur in the normal context of Gag expression from integrated proviruses of infected cells. To closely mimic the viral life cycle through a single round of infection and assembly, we began by infecting HeLa cells with VSV-G-pseudotyped FIV and HIV-1 particles containing env frameshifted but otherwise full-length genomes. After chromosomal integration, each lentivirus carries out the normal production of spliced and unspliced mRNAs and Gag/Gag-Pol production. Here again, FIV Gag accumulated in the nucleus after LMB treatment (Fig. 3C and D), but HIV-1 Gag did not and remained in the cytoplasm (Fig. 3G and H). The dramatic difference between FIV and HIV-1 is clear by comparing Fig. 3D and H. Also note that, in contrast to FIV Gag-CFP, virtually all native FIV Gag is imported into the nucleus (Fig. 3B and D), phenocopying a positive-control CRM1-dependent shuttle protein, Rev1.4-GFP-NES (23), was used as a positive control. Images are representative of at least three independent experiments for each virus.

Concomitantly with or shortly after budding, the Gag precursor p55 is cleaved by the virally encoded protease (PR) (28). To verify that the increase in nuclear fluorescence was due to the transport of intact Gag and not of the CA subdomain detected by the monoclonal antibody, we mutated the critical aspartic acid at position 30 of the protease catalytic domain (35) to a glycine residue in the FIV vector packaging plasmid pFP93 (44), generating pFP93Prm (Fig. 4A). A pol-deleted version that lacks all of the protease gene and the rest of pol except for a fragment of the integrase reading frame was also made (FP93pol). Western blotting showed that the cleavage of p55 Gag was abolished for both pFP93Prm and FP93pol (Fig. 4A[the blot also reconfirms, this time using antibody to Gag rather than to CFP as in Fig. 1I, that Gag-CFP does not fragment]). pFP93Prm and pFP93pol were transfected into HeLa cells, and immunofluorescence analysis was performed. LMB treatment resulted in the nuclear accumulation of both protease mutant FIV Gag polyproteins, similarly to Gag expressed from wild-type pFP93 (Fig. 4B). Moreover, the same results were also found when polyclonal anti-FIV sera from infected cats were used (data not shown).

In summary, HIV-1 Gag does not cycle through the nucleus. In stark contrast, experiments with Gag-CFP and with unlabeled Gag produced from either transfected plasmids or an integrated provirus demonstrate that FIV Gag transiently cycles through the nucleus, where it is retained when CRM1-mediated export is prevented.

CTE- versus Rev-mediated Gag mRNA export. How viral RNA traffics out of the nucleus and into the cytoplasm can influence the trafficking itinerary of the Gag protein translated from the RNA and modulate the efficiency of assembly (1, 51). We therefore investigated if changing the FIV RNA export pathway from a Rev/RRE-dependent mechanism to a CTE-dependent pathway affects the nuclear trafficking capability of FIV Gag-CFP. The FIV RRE and rev exons were replaced with four copies of the

FIG 3 Subcellular localization of unmodified Gag proteins analyzed in transfected and infected cells. (A to H) Both FIV (A to D) and HIV-1 (E to H) were tested. For transfections, HeLa cells were transfected with either FIV packaging plasmid pFP93 (A and B) or HIV-1 packaging plasmid CMVΔR8.91 (E and F). Alternatively, cells were infected with single-cycle VSV-G-pseudotyped env-lacking viruses for FIV infection (C and D) and HIV-1 infection (G and H). These single-cycle challenge viruses were generated in 293T cells by pseudotyping the viruses produced by pCT5efs and pHIV-1lac, respectively. (Note that pCT5efs, used here [C and D] and in feline cells in Fig. 6B produces FIV 34TF10, which is entirely of the wild type except for a 31-bp oligonucleotide inserted into env to frameshift it and allow single-cycle pseudotyping [29]). Forty-eight hours after transfection or infection, cells were left untreated or were treated with LMB for 3 h and then fixed, and indirect immunofluorescence was performed with monoclonal anti-FIV CA or monoclonal anti-HIV CA antibody and an anti-mouse Alexa Fluor 594-conjugated secondary antibody. (I and J) A CRM1-dependent shuttle protein, Rev1.4-GFP-NES (23), was used as a positive control. Images are representative of at least three independent experiments for each virus.
constitutive transport element (CTE) from MPMV, resulting in pFIVGag-CFP-4xCTE (Fig. 5). The FIV Gag-CFP thus generated was excluded from the nucleus (Fig. 5, left). LMB treatment resulted in nuclear and nucleolar translocations indistinguishable from those observed when FIV Gag-CFP was produced from a Rev-dependent RNA (compare Fig. 5, right, with 1C, E, J, and K). We conclude that the nuclear export pathway of the RNA does not determine the ability of FIV Gag to cycle through the nucleus.

Feline cells display FIV Gag nuclear shuttling, and infected cells contain nuclear Gag in the steady state. Cells from different mammalian species display various competences for specific lentiviral life cycle stages. Impediments arise from species-specific variations in restriction and dependency factors. The latter phenomenon is particularly evident in the case of HIV-1 gRNA trafficking and particle assembly in mouse cells, which proceed inefficiently (3, 38, 52). Indeed, variant residues in murine CRM1 itself have been reported to account for a substantial portion of this assembly-side block (50). We therefore considered that FIV Gag nuclear trafficking in human and African green monkey cells might proceed differently than in cells of the natural feline host. To test this possibility, we carried out transfection and infection of Crandell feline kidney (CrFK) cells. FIV Gag-CFP and unmodified FIV Gag were imaged by using direct excitation and immunofluorescence, respectively (Fig. 6).

This experiment not only demonstrated that FIV Gag cycles through the nucleus in feline cells but also showed that a substantial portion of FIV Gag can be seen to also accumulate in the nucleus when the CRM1 pathway is uninhibited (Fig. 6 and 7A). As in the human cell experiments (Fig. 3B and D and 4B), native Gag protein nuclear residence was nearly total after LMB treatment. Note that Fig. 6C shows feline cells that are productively infected at a low multiplicity of infection (MOI) (0.3) with wild-type, replication-competent FIV. Here the same LMB-induced nuclear concentration of Gag was seen (Fig. 6C). The representative syncytia shown demonstrate the approximately equivalent nuclear and cytoplasmic FIV Gag levels in the steady state (no LMB), while nuclear levels were much higher than cytoplasmic levels in infected cells after LMB treatment (Fig. 6C).

**Nucleolar localization.** As noted above, the FIV Gag-CFP protein consistently displayed nucleolar localization in most LMB-treated cells, while unmodified FIV Gag assessed by confocal im-

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**FIG 4** Subcellular localization of FIV Gag polyprotein. (A) pFP93Prm (protease catalytic domain mutant) and pFP93ΔPol (lacks pol) are illustrated (the segments containing gag-pol and the first exon of rev are shown). The bottom shows immunoblotting for these constructs along with pFIVgagCFP; in lane 2, pFP93, which encodes Gag-Pol (protease intact), was included. None indicates that no Gag DNA was transfected. (B) Indirect immunofluorescence showing subcellular distribution with and without LMB treatment. MAb PAK3-2C1, which recognizes an epitope in the CA domain of Gag, was the primary antibody (Ab) for immunofluorescence and immunoblotting. Mock-transfected cells (top) or pFP93-transfected cells incubated only with anti-mouse Alexa Fluor 594-conjugated secondary antibody (bottom) are shown at the right. Images are representative of at least three independent experiments.

**FIG 5** Export of Gag-CFP mRNA via the NFX1/NXT pathway does not alter CRM1-dependent nuclear cycling of Gag-CFP. Diagrammed at the top is pFIVGag-CFP-4xCTE, which encodes the FIV Gag-CFP fusion protein and in which the RRE and Rev exons of FIV are replaced with four copies of the CTE of MPMV. Photomicrographs show live-cell confocal images of HeLa or COS7 cells transfected with pFIVGag-CFP-4xCTE and treated or not with LMB for 3 h. Images are representative of at least three independent experiments.
munofluorescence displayed a clearly visible nucleolar location less frequently (ranging from 1 to 10% of cells in multiple experiments). To address the phenomenon of nucleolar targeting further and confirm the identity of the structures, we performed simultaneous immunofluorescence experiments with unmodified FIV Gag and the nucleolar marker fibrillarin in FIV-infected cells (Fig. 7A), pFP93-transfected cells (Fig. 7B), and pFP93/Apol-transfected cells (Fig. 7C). Fibrillarin, an rRNA 2'-O-methyltransferase, is associated with the U3, U8, and U13 small nuclear RNAs and is located in the dense fibrillar component (DFC) of the nucleolus. Some Gag was visible in the nuclei of FIV-infected cells in the absence of LMB (Fig. 7A); however, nucleolar concentration of Gag was observed infrequently. LMB again induced a major shift to nuclear-retained Gag (Fig. 7A). A nucleolar concentration was seen in a small and variable percentage of infected LMB-treated cells (5 to 10%), but in most infected cells, nucleoli had less Gag than the surrounding nucleoplasm (Fig. 7A, compare the cell in the inset photomicrograph with other cells). In cells transfected with pFP93 or pFP93/Apol, the nucleolar Gag concentration was more evident. In some transfections, such cells comprised 20 to 30% of the transfected monolayer, which may be related to the higher Gag expression levels achieved with transfection (Fig. 7B and C). In such cells, Gag was diffusely distributed within the nucleoli, with the signal not strictly confined to the fibrillarin-marked DFC (Fig. 7).

Assessment of potential nuclear localization and export signals. Since FIV Gag enters the nucleus, where it is retained if the CRM1 nuclear export pathway is inhibited, we hypothesized that it may contain classical nuclear localization and export signals. An analysis of the p55 Gag amino acid sequence with the Eukaryotic Linear Motif resource (11) revealed a potential leucine-rich NES.
in the MA subdomain between amino acids 53 and 66 (DIPETLDQRLV1C). However, mutations of the three leucines and the isoleucine at position 65 to alanines (DIPETADQRAVA(D)) did not result in a nuclear accumulation of Gag (data not shown), and therefore, this sequence does not function as an NES. We also identified a possible bipartite NES in MA [KR(X9)KSSK] with the cNLS Mapper program (31). Mutations of the four lysines and the arginine to alanines did not prevent nuclear import after LMB treatment, and therefore, this motif is not a functional NES (data not shown). To investigate other potential signals, we constructed pFIVGag-CFP mutants (Fig. 8). These mutants lack MA (ΔMA-CFP), CA (ΔC-A-CFP), or NCp2 (ΔNCp2-CFP). Live-cell confocal images are shown in Fig. 8A to H, and the ratio of nuclear/total MFI is shown in Fig. 8I. These experiments showed that GagΔMA-CFP behaved most similarly to Gag-CFP in the steady state, being present only in scant amounts in the nucleus in some cells and absent from the nucleus in others (Fig. 8A and C). This nuclear accumulation increased strongly after LMB treatment, although the intranuclear appearance differed somewhat from that of Gag-CFP (Fig. 8D and J). The CA deletion mutant at steady state approximately phenocopied the LMB-treated WT protein (compare Fig. 8B with E and F). Importantly, no further increase in the nuclear accumulation of the ΔCA protein was observed with LMB (Fig. 8E, F, and J). The subcellular localization of the protein lacking NCp2 was similarly indistinguishable in the presence or absence of LMB (Fig. 8G, H, and J). The NCp2-deleted protein distributed between the cytoplasm and the nucleus but was excluded from nucleoli. It was also indistinguishable from CFP alone (Fig. 1F and G and 8G and H). Thus, FIV NCp2 contains the nucleolar-targeting determinant of Gag-CFP.

To investigate this further, we analyzed individual Gag domains as CFP fusion proteins (Fig. 8K to Q). MA-CFP was diffusely present in both the cytoplasm and nucleus and was unaffected by LMB (Fig. 8K, L, and Q). CA-CFP localized to the cytoplasm and nucleus but was excluded from nucleoli, a distribution similar to those seen with MA-CA-CFP (i.e., ΔNCp2-CFP) and CFP alone (compare Fig. 8M, N, and Q with Fig. 8G, H, and J, and Fig. 1F and G). Suggesting that when it is expressed outside the viral context in this manner, CA is passively distributed, like CFP. NCp2-CFP showed a pronounced nuclear accumulation. NCp2 was also the only one of the three proteins to confer an accumulation of signal in nucleoli, confirming that it has the determinant for nucleolar targeting (Fig. 8O and P). Note also that this nucleolar targeting is infrequent with the full-length FIV Gag protein unless it is fused to CFP (compare Fig. 1C and E and 8A and B with 3B and D and 7A). There was no increase in the nuclear proportion of any of the three individually expressed Gag domains (MA-CFP, CA-CFP, or NCp2-CFP) upon LMB treatment (Fig. 8Q).

We conclude that each tested FIV Gag domain can enter the nucleus. Since no further increase in nuclear accumulation was observed when the CRM1 export pathway was inhibited, we also conclude that none of the individual domains demonstrates a classical NES. Importantly, CA-NCp2, CA, and NCp2 each differ markedly in the nucleocytoplasmic trafficking properties that they confer to a fluorescent protein (compare Fig. 8C and D with M and N and with O and P). Likewise, FIV Gag proteins lacking either CA or NCp2 did not retain the LMB-sensitive nuclear export properties of full-length Gag (Fig. 8E to H). A consideration of CA-NCp2 (i.e., ΔMA) in the context of all of the data shown in Fig. 1 to 8 indicates that the CRM1-dependent nuclear shuttling of FIV Gag is an emergent property of the combined CA-NCp2 portion that is not manifested by CA or NCp2 alone and which is unlikely to be mediated by a single linear peptide motif unless that motif is functionally unmasked in the context of the larger protein.

**DISCUSSION**

Our main discovery in this report, that a lentiviral Gag protein cycles through the nucleus, was unexpected. The HIV-1 MA domain was reported previously to have two NLSs (5, 21) and one NES (13), but multiple subsequent reports have shown that HIV-1 MA-GFP is excluded from the nucleus (2, 10) and that it does not contain a conventional NLS (15, 22). Two studies in the past year confirmed that HIV-1 Gag lacks a CRM1-dependent nuclear export signal (2, 18). In addition, HIV-1 Gag lacking the reported nuclear import and export signals can mediate efficient packaging (18). Our present results with HIV-1 Gag support the current consensus that a nuclear function of this protein in assembly can be excluded unless this occurs at a level that is below the detection limits of present methodologies. FIV is thus unique among the lentiviruses in having a Gag protein that undergoes nuclear cycling. In addition to expanding the spectrum of lentiviral Gag behaviors, these results raise the possibility that FIV genome encapsidation initiates in the nucleus. A comparative analysis of FIV and HIV-1 Gag/gRNA trafficking may help illuminate the spatial and temporal details of the encapsidation process in the cell.

There are two interesting precedents for retroviral Gag nuclear trafficking, both in nonlentiviruses. Parent and colleagues have shown in a series of studies that Rous sarcoma virus (RSV) Gag shuttles (6, 17, 20, 45–47), and experiments with RSV Gag mutants that do not undergo nuclear cycling provided evidence that this is needed for efficient gRNA encapsidation (17, 45). RSV Gag nuclear import is mediated by NLSs in MA and NC (45). In a recent study, the replacement of RSV MA with HIV-1 MA prevented detectable nuclear cycling of the resultant Gag chimera (2). The formation of infectious virus, as assessed by the ratio of transduced to transfected cells in a reporter virus plasmid-transfected monolayer, was not blocked, although it was reduced 50 to 70% (2). The binding of RSV Gag to the gRNA in the nucleus exposes an NES in the Gag p10 domain, which in turn promotes CRM1-RanGTP binding, which results in the nuclear export of the complex (20, 45, 47). The nuclear encapsidation process of this alpharetrovirus so far remains unique among retroviruses (40).

Prototype foamy virus (PFV) Gag has also been observed in the nuclear compartment (39, 48, 55). The mechanism is novel, since PFV Gag does not possess a functional NLS and does not translocate to the nucleus of interphase cells. Instead, it accesses the nucleus during mitosis and binds to the host cell chromatin, which is mediated by a chromatin binding site (CBS) in the NC domain (39). Recently, an NES in PFV Gag was described (43). The role of Gag nuclear trafficking in the PFV life cycle remains uncertain, with nuclear gRNA encapsidation not yet implicated.

Not only does FIV Gag undergo nuclear cycling, we observed that it can also be partially nuclear at the steady state when the CRM1 export pathway is not inhibited (Fig. 4B, 6B and C, and 7A) and also that virtually complete nuclear accumulation occurs in both human and feline cells when the CRM1 export pathway is inhibited (Fig. 3A to F, 4B, and 6C to F). The results suggest that
FIG 8 Subcellular localization of FIV Gag mutants. (A to H) Live-cell confocal images of HeLa cells transfected with 2 μg of pFIVGag-CFP (A and B), pFIVGagΔMA-CFP (left) or pFIVGagΔMA-mCherry (right) (C and D), pFIVGagΔCA-CFP (E and F), or pFIVGagΔNC-CFP (G and H) and treated or not with LMB for 3 h. (I) Immunoblotting confirmed the expression of single proteins of the predicted masses. The control (rightmost) lane was moved for presentation purposes by using editing software. (J) The MFI was measured in the nucleus and cytoplasm of 137 cells (10, 8, 21, 25, 11, 18, and 26 cells for the columns going from left to right, respectively), and the ratio of the MFI in the nucleus to the MFI in the total cell was determined and expressed as a percentage (mean ± standard deviation). (K to Q) Subcellular localization of FIV MA-CFP, CA-CFP, and NCP2-CFP. Transfected HeLa cells were treated or not with LMB for 3 h. The nuclear/total MFI (Q) was determined as described above for panel J for 158 cells (6, 17, 25, 15, 11, 16, 11, and 9 cells for columns from left to right, respectively). Images are representative of at least three independent experiments.
most, if not all, FIV Gag normally undergoes a nuclear cycle. The individual Gag domain–CFP fusions that we tested did not accumulate further in the nucleus with LMB treatment, and therefore none demonstrated a classical CRM1-dependent NES when tested in this manner. In contrast, LMB-sensitive nuclear export is conferred by CA-NCP2 but not by CA or NCP2 alone, and Gag deletion that lack either CA or NCP2 lose the nuclear export properties of full-length Gag. The nuclear concentration of CA-NCP2–CFP and CA-NCP2-mCherry in the presence of LMB (Fig. 8C and D) also implies that they are actively imported into the nucleus. Three general mechanisms can be considered. The first is that FIV Gag is a cargo that CRM1 engages by recognizing individually insufficient nuclear export determinants present in separate Gag domains (a multipartite signal). Such an export mechanism has been identified, for example, in Snurportin-1. Snurportin-1 binds CRM1 in a bipartite manner, with a leucine-rich NES located in the N terminus and a second signal in a basic surface on the nucleotide binding domain (12). The second possibility is that CA-NCP2 and/or the full-length Gag protein forms or exposes one or more noncanonical determinants that are not formed by individually expressed MA, CA, or NCP2. A third possibility would be a more indirect mechanism whereby Gag associates with another factor or macromolecular complex that shuttles. FIV p2 comprises the C-terminal 18 amino acids of Gag and contains the PSAP (late-domain) motif. p2 mediates Tsg101 interactions and particle release from the plasma membrane, analogously to HIV-1 p6 (37). While it remains possible that p2 contributes to full-length Gag nuclear cycling, it did not display such properties in the context of NCP2 (Fig. 8O and P) or ΔCA (Fig. 8E and F).

Note that fusion to FIV CA does not appreciably alter the nucleocytoplasmic distribution of CFP (compare Fig. 8M and N with 1F and G). These studies, in which CA was artificially expressed as a discrete protein, do not, of course, exclude that core-associated CA derived from gag cleavage during particle budding has important nuclear import functions in the postentry virion. Although the present work focused on the trafficking of de novo–expressed Gag, which is relevant to the assembly side of the life cycle, there is evidence that FIV and HIV-1 negotiate early events differently in some respects, including the use of nuclear entry pathways (32, 34, 54). Further comparative investigations of the roles of FIV versus HIV-1 Gag-derived virion structural proteins in preintegration trafficking steps may be informative.

The nucleolar targeting that we observed is of unclear significance. It is evidently dependent on NC, but the various ways in which this did or did not manifest in the various proteins that we tested may have implications. NC-conferred nucleolar localization was evident in multiple protein contexts (Fig. 1, 5, 7, and 8). The full-length Gag nucleolar concentration that became detectable (i.e., by reaching concentrations higher than those in the surrounding nucleoplasms) could be seen with all expression modes (virus infection, plasmid transfection, unmodified Gag, fluorescent protein–fused Gag, and in the presence and absence of functional protease). However, it was less frequent with Gag than with Gag–CFP. Fusion to mCherry, which is monomeric (49), produced reduced nucleolar localization compared to that of CFP. These observations, along with the fact that in LMB-treated cells, the fusion proteins are not fully nuclear, while unmodified FIV Gag is, suggest that the fusion of proteins to the C terminus of FIV Gag can modulate pertinent functions. This is an important area for further investigation.

When considered in the context of the effects of LMB, the steady-state accumulations of FIV Gag on the outer face of the nuclear envelope that we observed previously (30) are consistent with a model in which the CRM1-mediated FIV Gag nuclear export rate exceeds the Gag import rate. When expressed abundantly, FIV Gag may become relatively traffic jammed at the entry side, yielding a steady-state appearance with no visible nuclear Gag. Since fluorescent protein fusions to the FIV Gag C terminus appear to reduce its nuclear import, as a comparison of Fig. 1, 5, and 6A with 3, 4, and 6B and C in the present work shows, they may elevate this outer face stacking to a detectable level.

In summary, we demonstrate that FIV Gag is a nuclear shuttling protein that utilizes the CRM1 nuclear export pathway. We show this with transfected and infected cells, with fluorescent protein–fused Gag and native Gag, with human and feline cells, and under conditions where the presence of single full-length Gag proteins was verified. The experiments at the same time bolster the current consensus in refuting the notion of a nuclear phase of HIV-1 Gag. Our results raise the possibility that FIV encapsidation initiates in the nucleus, as in the prevailing RSV model. Because Gag is the driver of particle assembly, during which it participates in numerous sequential processes and protein–protein interactions, determining the specific role of Gag trafficking patterns in gRNA encapsidation remains technically challenging but represents an exciting future direction. The clear difference in nuclear cycling between HIV-1 and FIV Gag may provide utility for a comparative attack on the major unsolved problem of deciphering the spatiotemporal specifics of lentiviral gRNA encapsidation in the cell.

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REFERENCES

ERRATUM

Feline Immunodeficiency Virus Gag Is a Nuclear Shuttling Protein

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