Prototype Foamy Virus Gag Nuclear Localization: a Novel Pathway among Retroviruses^{▽†}

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Gag nuclear localization has long been recognized as a hallmark of foamy virus (FV) infection. Two required motifs, a chromatin-binding site (CBS) and a nuclear localization signal (NLS), both located in glycine-arginine-rich box II (GRII), have been described. However, the underlying mechanisms of Gag nuclear translocation are largely unknown. We analyzed prototype FV (PFV) Gag nuclear localization using a novel live-cell fluorescence microscopy assay. Furthermore, we characterized the nuclear localization route of Gag mutants tagged with the simian vacuolating virus 40-NLS (SV40-NLS) and also dissected the respective contributions of the CBS and the NLS. We found that PFV Gag does not translocate to the nucleus of interphase cells by NLS-mediated nuclear import and does not possess a functional NLS. PFV Gag nuclear localization occurred only by tethering to chromatin during mitosis. This mechanism was found for endogenously expressed Gag as well as for Gag delivered by infecting viral particles. Thereby, the CBS was absolutely essential, while the NLS was dispensable. Gag CBS-dependent nuclear localization was neither essential for infectivity nor necessary for Pol encapsidation. Interestingly, Gag localization was independent of the presence of Pol, Env, and viral RNA. The addition of a heterologous SV40-NLS resulted in the nuclear import of PFV Gag in interphase cells, rescued the nuclear localization deficiency but not the infectivity defect of a PFV Gag ΔGRII mutant, and did not enhance FV's ability to infect G₁/S-phase-arrested cells. Thus, PFV Gag nuclear localization follows a novel pathway among orthoretroviral Gag proteins.

Retroviral Gag proteins have numerous and very complex roles in the viral life cycle. These include functions in uncoating and reverse transcription (RT), interaction with cellular machinery, transcriptional regulation activities, the orchestration of viral assembly, and the encapsidation of other viral components (reviewed in reference 15). Furthermore, the retroviral Gag protein has essential functions in intracellular trafficking during both early and late stages of viral replication (reviewed in references 1, 38, and 51). There are reports that the Gag proteins of HIV type 1 (HIV-1), murine leukemia virus (MLV), prototype foamy virus (FV) (PFV), and Rous sarcoma virus (RSV) traffic to the nucleus (13, 34, 43, 45). Nuclear trafficking of Gag might occur at two steps of the viral life cycle. First, Gag (or subunits of Gag) is thought to be part of the preintegration complex (PIC) and might be involved in the PIC nuclear translocation process (reviewed in references 4 and 51). Second, the nascent Gag precursor transiently traffics through the nucleus during particle assembly (13, 34, 43, 45).

Retroviral Gag proteins contain multiple trafficking signals. For example, two nuclear localization signals (NLSs) (5, 19) and one nuclear export signal (NES) (13) have been described for the matrix (MA) domain of HIV-1 Gag. RSV Gag possesses two independent NLSs, one in the nucleocapsid (NC)

and one in the MA domain (7), as well as an NES in p10 (44). In contrast, NLS and NES elements have not been identified in MLV Gag.

Little is known about the functional role of retroviral Gag nuclear localization. For HIV-1, it was suggested that the NLS participates in the targeting of the PIC to the nucleus, whereas during virus production the NES counteracts the NLS in order to maintain Gag in the cytoplasm (13). Furthermore, it has been suggested that PFV Gag tethers the viral genome to the host cell genome, thereby facilitating integration (reviewed in reference 9). For RSV Gag, nuclear trafficking is necessary for efficient incorporation of viral RNA (vRNA) into particles (16).

Numerous trafficking processes have also been demonstrated for FV Gag. Spumaviruses or FVs are the only genus of the *Spumaretrovirinae*, the second subfamily of the retroviruses, along with the *Orthoretrovirinae* (41). The best-characterized isolate is PFV. Several major features of the spumavirus replication strategy are unique among retroviruses, and many are clearly reminiscent of another family of reverse transcriptase-encoding viruses, the hepadnaviruses (reviewed in references 29 and 41). As with other retroviruses, PFV Gag constitutes the major structural component of the viral particle and engages in complex interactions with the intracellular trafficking machinery.

Incoming FVs traffic along the microtubule (MT) network to the microtubule-organizing center (MTOC) (42). Therefore, Gag interacts with light chain 8 of the MT-dependent dynein motor (36). At the MTOC, uncoating and capsid disassembly occur through cleavage of the Gag structural protein by viral and cellular proteases (25). Gag is part of the PFV PIC, and it has been suggested that PFV Gag translocates to the cell

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nucleus and facilitates integration by tethering the viral DNA genome to the host cell chromatin (52). How the PIC localizes to the nucleus remains poorly understood.

It is clear that FV replication is cell cycle dependent, because FV gene expression requires mitosis (35, 51, 54). However, the PFV genome is found in the nucleus of cells arrested in the G₁/S phase (35, 42). Nuclear targeting of simian foamy virus (SFV) of macaques (SFVmac) Gag and the viral genome in G₁/S-phase-arrested cells was shown to be dependent on intact integrase (IN) (31). During the late stages of infection, *de novo*-translated PFV Gag is again targeted to the MTOC, where it preassembles into capsids. Interestingly, nascent PFV Gag was also reported to undergo transient trafficking through the nucleus (45).

PFV Gag nuclear localization, therefore, occurs at least twice during the viral replication cycle, once early with the incoming PIC and once late when nascent Gag is expressed. At least part of the required motif resides in glycine-arginine-rich box II (GRII), for which actually two different motifs have been described. An NLS was identified in the C-terminal part of GRII (45, 57), and a chromatin-binding site (CBS), which mediates interaction of Gag with H2A/H2B core histones, was identified in the N-terminal part (52). Parts of GRII are well conserved among all FV Gag proteins and, with the exception of feline FV (FFV) Gag, all known FV Gag proteins localize to the cell nucleus (3, 24, 30, 36, 53, 55). Very recently, a NES was described for PFV Gag (40).

Currently, functional explanations for nuclear localization of PFV Gag remain speculative. In this regard, we sought to determine how and at which stage of the viral replication cycle the individual motifs exert their function. To accomplish this, we took advantage of the newly available autofluorescent protein (AFP)-tagged Gag (and mutants thereof) (50), which we analyzed using a live-cell fluorescence microscopy assay. In contrast to earlier studies, we found that PFV Gag does not possess a functional NLS enabling nuclear import in inter-phase nuclei, but rather that PFV Gag nuclear localization occurs only by chromatin association through the CBS during mitosis. These results reveal a novel localization pathway among retroviral Gag proteins and suggest novel functionality.

MATERIALS AND METHODS

Cells and culture conditions. The human kidney cell line 293T (11), the human fibrosarcoma cell line HT1080 (39), the human epithelium HeLa cell line (46), the human epithelium T-REx-HeLa cell line (Invitrogen, Carlsbad), and the retinal pigment epithelium cell line ARPE-19 (12) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. HeLa, T-REx-HeLa, and ARPE-19 cells used for confocal laser scanning microscopy or live-cell imaging were cultivated in phenol red-free media.

Recombinant plasmid DNAs. A 4-component PFV vector system consisting of the expression vectors pcoPG4 (PFV Gag), pcoPE (PFV Env), pcoPP or pcziPol, (PFV Pol), and the enhanced green fluorescent protein (eGFP)-expressing PFV transfer vector pMD9 has been described previously (20, 33, 50). Due to bio-safety issues, a previously described variant PFV Pol expression construct with catalytically inactive reverse transcriptase, pcoPP2 (Pol iRT) (32, 33), was used for live-cell imaging experiments employing the 4-component PFV vector system. For microscopy analysis, the β -galactosidase (LacZ)-expressing PFV transfer vector pMD11 (20) was used instead of eGFP-expressing pMD9.

Individual PFV Gag constructs used in this study are depicted in Fig. 3A and 6A. Chemically synthesized oligonucleotides, coding for the peptide sequence of a duplicated simian vacuolating virus 40 (SV40)-NLS separated by a single glycine residue, were introduced in the previously described Gag expression

vectors pcoPG4 (wt), pcoPG4 CeYFP (CeYFP wt) (50), pcoPG4 Δ GRII (Δ GRII), and pcoPG4 Δ GRII CeYFP (CeYFP Δ GRII) (33). Together with a flexible glycine-serine linker, the SV40-NLS peptide was added to the C terminus of the respective Gag proteins to obtain the pcoPG4 SV40-NLS (wt SV40-NLS 1), pcoPG4 Δ GRII SV40-NLS (Δ GRII SV40-NLS 1), pcoPG4 CeYFP SV40-NLS (wt CeYFP SV40-NLS 1), and pcoPG4 Δ GRII CeYFP SV40-NLS (Δ GRII CeYFP SV40-NLS 1) constructs. Furthermore, the GRII sequence was replaced by the SV40-NLS peptide in the constructs pcoPG4 GRII/SV40-NLS (Δ GRII SV40-NLS 2) and pcoPG4 CeYFP GRII/SV40-NLS (Δ GRII CeYFP SV40-NLS 2). The pcoPG4 Δ NLS (Δ NLS), pcoPG4 CeYFP Δ NLS (CeYFP Δ NLS), pcoPG4 Δ CBS (Δ CBS), and pcoPG4 CeYFP Δ CBS (CeYFP Δ CBS) constructs were generated by introducing chemically synthesized oligonucleotides coding for the peptide sequence of the NLS of GRII or the CBS of GRII, respectively, in place of GRII. The pLV7 (EF1a MCS ZeoW) construct was generated based on the pLenti4/TO/V5-DEST lentiviral vector (Invitrogen, Carlsbad, CA). The original SV40 early promoter was replaced by an EF1a promoter, and the woodchuck hepatitis posttranscriptional regulatory element was inserted downstream of the Zeocin resistance gene. Additionally, the original *attR1* site, the chloramphenicol resistance gene, the *ccdB* gene, the *attR2* site, and the V5 epitope were replaced by multiple cloning sites (SpeI, SmaI, NheI, XhoI, PmeI, and EcoRI). The pLV7 PG construct was generated by excision of the expression-optimized Gag gene from pcoPG4 (50) and subsequent ligation of the insert into pLV7 (EF1a MCS ZeoW) using the SmaI and XhoI restriction sites. The pLV7 PG CeYFP construct was generated by excision of the expression-optimized Gag gene with a C-terminal enhanced yellow fluorescent protein (eYFP) (CeYFP) tag from pcoPG4 CeYFP (50) and subsequent ligation of the insert into pLV7 (EF1a MCS ZeoW) using the SmaI and XhoI restriction sites. All constructs were verified by sequencing analysis. Primer sequences and additional details are available upon request.

Transfection and virus production. Cell culture supernatants containing recombinant viral particles were generated by transfection of 293T cells using polyethylenimine (PEI) or the PolyFect transfection reagent as described previously (26, 28, 33). For subsequent Western blot analysis, the supernatant generated by transient transfection was harvested, passed through a 0.45- μ m filter, and centrifuged at 4°C and 25,000 rpm for 3 h in an SW40 or SW28 rotor (Beckman) through a 20% sucrose cushion. The particulate material was resuspended in phosphate-buffered saline (PBS).

Lentivirus-mediated target cell transduction. 293T cells were cotransfected with the HIV-1 Gag/Pol, Tat, Rev expression plasmid pCD/NL-BH* (58), the vesicular stomatitis virus glycoprotein encoding vector pczVSV-G (37), and the transfer vector pLV7 PG or pLV7 PG CeYFP by the use of PEI transfection reagent to generate cell culture supernatants containing recombinant lentiviral particles for stable transduction. At 32 h posttransfection (p.t.), the supernatant was exchanged for 6 ml of fresh cell culture medium. The supernatant was harvested at 48 h p.t., and 3 ml of supernatant and serial dilutions thereof were used for infection of 6×10^4 HeLa or T-REx-HeLa cells, seeded 24 h prior to infection. The cultivation medium was supplemented with 200 μ g/ml Zeocin. Pools of transduced cells were selected by replacing Zeocin-containing medium every 3 days until untransduced control cells had died.

Infectivity analysis. Transduction efficiency of recombinant, eGFP-expressing PFV vector particles was analyzed by fluorescence marker gene transfer assay as described previously (48). All transduction experiments were performed at least three times. In each independent experiment, the values obtained with the wild-type (wt) construct pcoPG4 were arbitrarily set to 100%, and values obtained with other constructs were normalized as a percentage of the wild-type values.

Cell cycle arrest. HT1080 cells were seeded at a density of 1×10^5 cells/well in 12-well plates 24 h prior to infection and treated with 4 μ g/ml aphidicolin 16 h prior to infection to arrest cells at the G₁/S phase of the cell cycle (6). Aphidicolin-containing medium was replenished at the time of infection. Transduction efficiency of infected arrested cells was analyzed 24 h p.t. by a fluorescence marker-gene transfer assay. Cell cycle arrest with aphidicolin was verified by flow cytometry for DNA content by propidium iodide staining. The data were analyzed using FlowJo software (Tree Star).

Small-scale isolation of chromatin. Biochemical fractionation of transiently transfected HeLa cells or induced T-REx-HeLa cells was performed as described previously (56). Briefly, cells were collected, washed with PBS, and resuspended at 4×10^7 cells/ml in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Triton X-100 was added (0.1% final concentration), the cells were incubated on ice for 8 min, and nuclei (fraction P1) were collected by centrifugation (5 min, $1,300 \times g$, 4°C). The supernatant (fraction S1) was clarified by high-speed centrifugation (5 min, $20,000 \times g$, 4°C), and the super-

FIG. 1. PFV Gag nuclear localization. (A) Merged live-cell microscopy images illustrating PFV Gag nuclear localization in transfected cells. eYFP-tagged Gag (green channel) and monomeric red fluorescent protein (mRFP)-tagged histone 2B (red channel) were cotransfected into HeLa cells. Microscopy images were taken starting 12 h p.t. every 15 min for 48 h. (B) Live-cell microscopy images illustrating PFV Gag nuclear localization in stable expressing cells. T-Rex-HeLa Gag CeYFP cells were induced with 1 μ g/ml tetracycline. Microscopy images were taken starting right after induction every 15 min for 56.5 h. (C) Live-cell microscopy images illustrating PFV Gag nuclear localization upon infection. Viral particles composed of Gag CeYFP were produced from transfected 293T cells and adhered at low temperature to HeLa cells before the temperature was shifted to 37°C. Microscopy images were taken starting right after infection every 15 min for 13.5 h. The images illustrate the cell when the Gag signal was first detected and right before, during, and after cell division and the daughter cells after completion of cell division. For the underlying complete image sequences of the observation period, see Videos S1, S2, and S3 in the supplemental material. Scale bar: 20 μ m.

natant (fraction S2) was collected. The P1 nuclei were washed once in buffer A and were lysed for 30 min in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and phenylmethylsulfonyl fluoride), and insoluble chromatin (fraction P3) and soluble (fraction S3) fractions were separated by centrifugation (5 min, 1,700 $\times$ g, 4°C). The P3 fraction was washed once with buffer B, resuspended in protein sample buffer, and boiled for 10 min.

Western blot analysis. Cells from a single transfected 100-mm cell culture dish were lysed in detergent-containing buffer, and the lysates were subsequently centrifuged through a QIAshredder column (Qiagen). Protein samples from cellular lysates or purified particulate material were separated by SDS-PAGE on a 10% polyacrylamide gel and analyzed by immunoblotting as described previously (27). Polyclonal rabbit antisera specific for PFV Gag (47) or the amino acids (aa) 1 to 86 of the PFV Env leader peptide (LP), (27) as well as hybridoma supernatants specific for PFV RT (clone 15E10) or PFV IN (clone 3E11) (22) were employed. After incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody, the blots were developed with Immobilon Western HRP substrate. The chemiluminescence signal was digitally recorded using a LAS-3000 imager.

Confocal microscopy. HeLa cells (6×10^4 cells per well on coverslips in 12-well plates) were transfected with 0.1 μ g Gag C-terminal eYFP fusion expression plasmid (pcoPG4 CeYFP or mutants thereof as indicated) using FuGENE HD transfection reagent (Roche). The cells were washed with cold PBS and fixed with 3% paraformaldehyde at 12 or 48 h p.t. After fixation the cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) for 5 min. Finally, the cells were covered with Mowiol.

Confocal laser scanning images were obtained on a Zeiss LSM 510 using a Zeiss Apochromat 63 $\times$, 1.4-numerical aperture (NA), oil immersion objective with the excitation light of a diode laser at 405 nm or an argon laser at 488 nm, reflected by a dichroic mirror (HFT 405/488/561). The fluorescence signal was recollected by the same objective, split by a dichroic mirror (NFT 490), passed through a 460/50 or a 520/35 filter, and subsequently measured by a photomultiplier tube (PMT). Confocal geometry was ensured by a 1-Airy unit pinhole in front of the PMT. Fluorescence images were evaluated using ImageJ software.

Live-cell microscopy analysis. To analyze nascent Gag proteins, HeLa or ARPE-19 cells (4×10^4 cells per well in a μ -Slide 8 well [ibidi]) were cotransfected with 0.05 μ g AFP-tagged Gag expression vectors (pcoPG4 CmCherry, pcoPG4 NeYFP, pcoPG4 CeYFP, or mutants thereof as indicated), or together with Env (pcoPE) and Pol iRT (pcoPP2) expression vectors, and the transfer vector (pMD11), at a ratio of 4:1:2:28, as well as 0.2 μ g pH2b-mRFP (23) using

FuGENE HD transfection reagents (Roche). To analyze Gag proteins in stable, transduced tetracycline-inducible cells (T-Rex-HeLa Gag CeYFP), 4×10^4 cells per well were seeded in a μ -Slide 8 well and induced with 1 μ g/ml tetracycline 6 h later. For live-cell analysis of incoming Gag protein, HeLa cells were seeded at a density of 2×10^4 cells/well into a μ -Slide 8 well. After 24 h, fluorescent PFV particle preparations were allowed to bind to cells at 12°C for 30 min. Subsequently, the cells were washed with cold PBS and supplemented with Leibovitz's L-15 medium (Invitrogen).

Live-cell imaging was performed at 37°C on a Nikon TE2000-E using a Lambda DG-4 with 470/30 and 575/20 excitation filters and a Nikon CFI planapochromat 40 $\times$, C, 0.95-NA objective. The fluorescence signal was recollected by the same objective, passed through a 517/55 LP590 dual-band filter, and subsequently measured by a Cool snap HQ charge-coupled-device (CCD) camera. Image sequences were evaluated using NIS-Elements or ImageJ software.

RESULTS

Nascent Gag enters the cell nucleus during mitosis. To determine the intracellular localization of nascent Gag, cells were transfected with AFP-tagged Gag expression plasmid. Live-cell microscopy of transfected cells from 12 to 48 h p.t. revealed that Gag was first homogeneously localized in the cytoplasm of interphase cells and excluded from the cell nucleus, with sporadic accumulations in the perinuclear region. There, Gag colocalized with DsRed-fused γ -tubulin (17) (data not shown). Neither the trafficking of Gag to the nucleus nor an accumulation of Gag in the nucleus was observed. Only during mitosis, putatively during nuclear envelope breakdown, Gag colocalized with the cells' chromatin, was distributed to the newly forming daughter nuclei by tethering to the chromatin, and finally, exclusively localized in the nuclei of the daughter cells (Fig. 1A; see also Video S1 in the supplemental material). Occasionally, a minor residual amount of Gag could

FIG. 2. Inducible PFV Gag eYFP cell line. Representative Western blot analysis of cell lysates from T-REx-HeLa Gag CeYFP cells induced with 1 μ g/ml tetracycline for 0 h (lane 1), 24 h (lane 2), or 48 h (lane 3). HeLa cell lysates from 48 h p.t., transfected with pcoPG4 (lane 4), pcoPG4 CeYFP (lane 5), or pcDNA3.1 zeo+ (lane 6) are shown as controls. Immunoblots were probed with an anti-PFV Gag (α -Gag) specific antiserum.

still be detected in the cytoplasm at the MTOC, right after cell division (data not shown).

After being localized to the nucleus during mitosis, Gag was found exclusively in the nucleus in most cells. Even during later cell divisions, when the nuclear envelope again disassembled, Gag remained associated, and cotrafficked, with the chromatin in these cells (data not shown). Only in few cells, Gag reaccumulated in the cytoplasm after a first mitosis and the associated Gag nuclear localization. This accumulation seemed to be the result of continued expression of nascent Gag. However, from this analysis, we cannot exclude protein export from the nucleus.

PFV Gag intracellular distribution was independent of the type of AFP (eYFP, eGFP, or mCherry) fused to Gag, of whether the tag was an N- or C-terminal fusion, of which target cells were used, and most notably, of whether Gag was expressed solely or together with Pol, Env, and transfer vector (data not shown).

Analysis of Gag localization in transduced cells. Protein expression from transfected plasmids is only transient, and the initiation as well as the level of expression in single cells is quite variable. Therefore, we used lentiviral transduction to obtain T-REx-HeLa cells, which contain a stable integration of the *gag CeYFP* gene under the control of the tetracycline-inducible cytomegalovirus virus (CMV)/TO promoter. Gag expression in these cells was detected biochemically and by fluorescence microscopy only upon induction with tetracycline (Fig. 2).

When we analyzed the cells by live-cell microscopy, the overall signal intensity was lower but less variable than in transiently transfected cells. The spatiotemporal intracellular Gag localization was similar to that of transiently transfected cells. Initial Gag expression was detectable 3 h postinduction (p.ind.). Gag was homogeneously distributed in the cytoplasm until 5 h p.ind., after which it started to accumulate in the perinuclear region. No import of Gag in the intact cell nucleus was observed, and Gag entered the cell nucleus only during mitosis, as it associated with the chromatin during nuclear envelope dissolution; as the nuclear envelope reformed, Gag bound to chromatin was enclosed within the nucleus (Fig. 1B; see also Video S2 in the supplemental material). After cell division, Gag started to accumulate in the cytoplasm again, probably as a result of the continued expression of nascent

Gag. If tetracycline was washed away from the cells, Gag expression stopped and no reaccumulation of Gag in the cytoplasm could be observed after subsequent cell divisions (data not shown). Thus, the data obtained from Gag expressed from an integrated genome confirmed the data obtained from transient expression.

Incoming Gag enters the cell nucleus during mitosis. PFV Gag of incoming viral particles is thought to localize to the cell nucleus (reviewed in reference 9). To explore how Gag from incoming viral particles traffics in the infected cell, we performed live-cell imaging of HeLa cells infected with viral particle preparations (multiplicity of infection [MOI], 100) purified from cell culture supernatants of 293T cells transfected with pcoPG4 CeYFP, pcoPE, pcoPP2, and pMD11, resulting in the generation of PFV particles with eYFP-tagged capsids, enzymatically inactive RT, and a LacZ-expressing vector genome.

After loading at low temperature, Gag eYFP-labeled particles were located at the cell surface. Two hours after shifting the temperature to 37°C, we observed Gag exclusively in the cytoplasm. At this point, we did not observe Gag in the nucleus, and Gag was no longer associated with the plasma membrane. We also observed Gag accumulation at a punctuate structure close to the nucleus, which appeared to be the MTOC, as Gag there colocalized with γ -tubulin (K. Stirnagel, A. Dupont, D. Schupp, F. Perrohon, E. Müllers, D. Lindemann, and D. Lamb, unpublished data). Over time, Gag accumulated almost exclusively at the MTOC. Sometimes a division of the MTOC about 30 min prior to the onset of cell division could be seen. Upon mitosis, Gag entered the cell nucleus by associating with the cellular chromatin, thereby being transported into the newly forming daughter nuclei (Fig. 1C; see also Video S3 in the supplemental material).

Thus, Gag from incoming viral particles first trafficked to and accumulated at a structure in close proximity to the cell nucleus, which we confirmed to be the MTOC (Stirnagel et al., unpublished). Furthermore, similar to endogenously expressed Gag, exogenously delivered Gag also entered the nucleus only during cell division by association and colocalization with cellular chromatin.

A heterologous NLS is sufficient to localize Gag to the nucleus. Because we did not observe the transport of PFV Gag through an intact nuclear membrane, we asked whether Gag could be transported into the nucleus during interphase in an NLS-dependent manner. Therefore, we introduced an SV40-NLS sequence into the PFV Gag wt (wt SV40-NLS 1 mutant) and also into the PFV Gag Δ GR11 mutant (Δ GR11 SV40-NLS1) (Fig. 3A). To analyze intracellular distribution by fluorescence microscopy, the SV40-NLS-containing mutants were also established as C-terminal eYFP fusion proteins. HeLa cells transfected with these eYFP-tagged Gag expression constructs were fixed 12 or 48 h p.t., stained with DAPI, and analyzed by confocal microscopy (Fig. 3B).

At 12 h p.t., wt Gag and the Δ GR11 mutant were found only in the cytoplasm, while all mutants that contained an SV40-NLS were found in the cell nucleus (Fig. 3B and C, 12 h p.t.). That is, Δ GR11 SV40-NLS 1 was found exclusively in the nucleus, while the wt SV40-NLS 1 and the Δ GR11 SV40-NLS 2 mutants were also found partly in the cytoplasm. Interestingly, the wt SV40-NLS 1 mutant exhibited nuclear rim staining,

FIG. 3. Schematic illustration and intracellular localization of PFV Gag SV40-NLS mutants. (A) Schematic outline of the generated PFV Gag SV40-NLS mutant constructs established in wild-type and C-terminal eYFP fusion contexts. The three Gag GR boxes are indicated as gray boxes. The p68/p71 PFV Gag cleavage site is shown as a dashed line. NLS, nuclear localization signal; L, glycine-serine linker. (B) HeLa cells were transfected with expression plasmids for C-terminal eYFP-tagged Gag pcoPG4 CeYFP (row corresponding to wt in panel A) or pcoPG4 CeYFP Δ GRII (Δ GRII). (C) HeLa cells were transfected with expression plasmids for C-terminal eYFP-tagged Gag pcoPG4 CeYFP SV40-NLS (wt SV40-NLS 1), pcoPG4 CeYFP Δ GRII SV40-NLS (Δ GRII SV40-NLS 1), or pcoPG4 CeYFP GRII/SV40-NLS (Δ GRII SV40-NLS 2). The samples were fixed at 12 or 48 h p.t., stained with DAPI, covered in Mowiol, and analyzed by confocal laser scanning microscopy. The merge column shows a merged image of the eYFP (green) and the DAPI (red) channels. The eYFP (Gag) column shows the obtained signal in the eYFP channel. Scale bar: 10 μ m. (D) Representative Western blot analysis of fractionated cell lysates from HeLa cells transiently transfected for 12 h or 48 h with either pcoPG4 (Gag wt), pcoPG4 Δ GRII (Gag Δ GRII), or pcoPG4 SV40-NLS (Gag wt SV40-NLS 1). Immunoblots were probed with an anti-PFV Gag (α -Gag)-specific hybridoma supernatant or an anti-GAPDH (α -GAPDH) or anti-H3 (α -Histone)-specific antibody (Sigma-Aldrich).

while the Δ GRII SV40-NLS 1 and the Δ GRII SV40-NLS 2 mutants exhibited nucleolar staining. At 48 h p.t., Gag was found in cell nuclei for all mutants, except the Δ GRII mutant (Fig. 3B and C, 48 h p.t.). Again, the wt SV40-NLS 1 mutant showed prominent nuclear rim staining, while the Δ GRII SV40-NLS 1 and the Δ GRII SV40-NLS 2 mutants exhibited nucleolar staining. The cytoplasmic staining of the wt SV40-

NLS 1 and Δ GRII SV40-NLS 2 mutants was similar to that of the wt, while the Δ GRII SV40-NLS 1 mutant showed hardly any Gag in the cytoplasm. When we analyzed cells undergoing division, Gag colocalized with the chromosomes in the wt and the wt SV40-NLS 1 sample, because both constructs contain an intact GRII. No chromatin colocalization was observed for the Δ GRII mutant.

FIG. 4. Cellular and particle-associated protein expression analysis and infectivity analysis of the PFV Gag SV40-NLS and GRII box mutant constructs. (A and B) Representative Western blot analysis of 293T cell lysates (cell) and viral particles (virus) purified by ultracentrifugation through 20% sucrose for Gag SV40-NLS and GRII box mutants. PFV proteins were detected using an anti-PFV Gag (α -Gag)- or anti-PFV Env LP (α -LP)-specific antibody or an anti-PFV Pol

A weak colocalization with the chromosomes and a diffuse cellular staining was observed for the Δ GRII SV40-NLS 1 and Δ GRII SV40-NLS 2 mutants (Fig. 3B and C, 48 h p.t.). As this was not observed with the Δ GRII mutant, it seemed to result from the added SV40-NLS.

Similar spatiotemporal localization was observed by live-cell imaging analysis (data not shown). Furthermore, the results were confirmed by small-scale chromatin purification of transiently transfected HeLa cells. At 12 h p.t., wt Gag and the Δ GRII mutant were found only in the cytoplasmic fraction, whereas the wt SV40-NLS 1 mutant was already found mainly in the nuclear fractions (Fig. 3D, 12 h p.t.). At 48 h, wt Gag and the wt SV40-NLS 1 mutant were also found mainly in the nuclear fractions, whereas the Δ GRII mutant was still found exclusively in the cytoplasmic fraction (Fig. 3D, 48 h p.t.).

Thus, while authentic PFV Gag nuclear localization is strictly dependent on cell division, Gag can be localized to the nucleus by a heterologous NLS during interphase.

Artificial nuclear localization is insufficient to rescue viral infectivity. PFV Gag mutants that lack GRII and, therefore, nuclear localization are also characterized by reduced infectivity and defects in Pol packaging (33, 45, 52, 57). To examine whether nuclear targeting of PFV Gag by an SV40-NLS can rescue these defects or whether Gag nuclear targeting by an alternative import mechanism rather impairs infectivity, viral particles were produced from 293T cells transfected with expression vectors for the Gag wt, Gag Δ GRII, or the Gag SV40-NLS mutants, together with Env (pcoPE), Pol (pcziPol), and a transfer vector (pMD9). Subsequently, the viral supernatants were analyzed for viral protein composition and infectivity.

Our results showed wt-like cellular expression for all Gag mutants (Fig. 4A, lanes 1 to 5). Cellular expression levels of Env and Pol were unaffected (data not shown). All mutants released wt-like amounts of viral particles. However, reduced amounts of Pol and the diminished processing of Gag were observed for the Gag Δ GRII mutant, the Gag Δ GRII SV40-NLS 2 mutant, and particularly the Gag Δ GRII SV40-NLS 1 mutant. Interestingly, even the Gag wt SV40-NLS 1 mutant, with its intact GRII, showed similarly decreased Pol packaging (Fig. 4B, lanes 1 to 5, anti-Gag and anti-PR/RT anti-IN).

Relative infectivity was 5% for Δ GRII, 16% for wt SV40-

PR/RT (α -PR/RT)- or anti-PFV Pol IN (α -IN)-specific hybridoma supernatant. 293T cells were cotransfected with pMD9, pcziPol, pcoPE, and pcoPG4 SV40-NLS (lane 1, wt SV40-NLS 1), pcoPG4 Δ GRII SV40-NLS (lane 2, Δ GRII SV40-NLS 1), pcoPG4 GRII/SV40-NLS (lane 3, Δ GRII SV40-NLS 2), pcoPG4 (lane 4, wt), pcoPG4 Δ GRII (lane 5, Δ GRII), pcoPG4 Δ NLS (lane 7, Δ NLS), or pcoPG4 Δ CBS (lane 8, Δ CBS). Control cells were transfected with pcDNA3.1 zeo+ only (lane 6, mock). (C) PFV particle-containing cell culture supernatants were generated by transient transfection of 293T cells using the 4-component PFV vector system. Relative infectivity of extracellular cell culture supernatants using the eGFP marker gene transfer assay was measured 3 days p.i. The values obtained using wild-type PFV Gag expression plasmids were arbitrarily set to 100%. Means and standard deviations from three independent experiments are shown. Differences between means of wt and the individual mutants were analyzed by Welch's *t* test and found very significant ($P < 0.01$) for all mutants.

FIG. 5. Infectivity of the PFV Gag and Gag wt SV40-NLS 1 in aphidicolin-treated cells. PFV particle containing cell culture supernatants were generated by transient transfection of 293T cells using the 4-component PFV vector system. The supernatants were used to infect HT1080 cells treated with 4 μ g/ml aphidicolin for 16 h prior and continuously upon infection (Aphidicolin) or untreated cells (Control). Relative infectivity of extracellular cell culture supernatants using the eGFP marker gene transfer assay was measured 1 day p.i. The value obtained using wild-type PFV Gag expression plasmid was arbitrarily set to 100%. Means from a representative experiment conducted in duplicates are shown. 293T cells were cotransfected with pMD9, pcoPP, pcoPE, and pcoPG4 (wt) or pcoPG4 SV40-NLS (wt SV40-NLS 1). Control cells were transfected with pcDNA3.1 zeo+ only (mock).

NLS 1, 0.5% for Δ GRII SV40-NLS 1, and 0.1% for Δ GRII SV40-NLS 2, compared to that for the wt (Fig. 4C, lanes 1 to 5).

Thus, heterologous NLS-dependent nuclear localization of PFV Gag seems to rescue neither Pol packaging nor the infectivity defect of the Gag Δ GRII mutant. Addition of an SV40-NLS to Gag rather leads to an additional 10- to 50-fold reduction in infectivity in the Δ GRII context and to an about 7-fold reduction if the SV40-NLS was added to wt Gag. Interestingly, the observed reduction in infectivity appeared to correlate with the diminished amount of Pol packaged for each of the mutant Gag proteins.

Heterologous nuclear localization does not improve the ability of PFV to infect nondividing cells. In contrast to lentiviral vectors, FV vectors are unable to transduce cells arrested by aphidicolin (54). To investigate if heterologous NLS-dependent nuclear targeting of PFV Gag improves infection of G_1/S phase-arrested cells, supernatants with wt or Gag wt SV40-NLS 1 mutant particles were used to infect HT1080 cells, which had been arrested by aphidicolin or left untreated. Viral titers on aphidicolin-treated HT1080 cells were similar, about 50-fold reduced for both the wt and the Gag wt SV40-NLS 1 mutant in comparison to those on untreated cells (Fig. 5). Thus, we did not find efficient infection of aphidicolin-treated cells by PFV, and fusion of a heterologous NLS to Gag did not improve the ability of PFV to infect these nondividing cells.

Respective contributions of GRII CBS and NLS subdomains to nuclear localization. Two different motifs, a CBS in the N-terminal part of GRII and an NLS in the C-terminal part, were reported to be responsible for PFV Gag nuclear localization (45, 52, 57). To explore the respective contributions of

each motif in the context of p71^{Gag}, we expressed in HeLa cells eYFP-tagged Gag mutants lacking either the described NLS subdomain (Δ NLS) or the described CBS subdomain (Δ CBS). The results were compared to those for authentic Gag (wt) and the Δ GRII mutant (Δ GRII) (Fig. 6A). The cells were fixed 12 or 48 h p.t. and stained with DAPI.

Confocal microscopy analysis showed that wt Gag and all Gag mutants were localized solely to the cytoplasm at 12 h p.t. At 48 h p.t. nuclear localization was observed for the wt and the Δ NLS mutant samples, but not for the Δ GRII and the Δ CBS mutant samples. When we then analyzed cells in division, Gag colocalized with the chromosomes in the wt and the Δ NLS mutant sample but not in the Δ GRII mutant and the Δ CBS mutant samples (Fig. 6B). Live-cell imaging of transiently transfected cells revealed similar results (data not shown).

In summary, the Gag mutant lacking the NLS sequence (Δ NLS) displayed a wild-type like intracellular distribution, while the mutant lacking the CBS sequence (Δ CBS) had a subcellular localization phenotype similar to that of Gag Δ GRII. Thus, the CBS was necessary and sufficient for colocalization of Gag with chromatin and nuclear localization, while the NLS was dispensable.

Respective contributions of GRII CBS and NLS subdomains to particle infectivity and Pol packaging. PFV Gag mutants with deletions in GRII are associated with a reduced infectious titer and diminished Pol packaging (33, 49, 52, 57). To explore how the CBS and NLS contribute to this phenotype, viral particles were produced by transfection of 293T cells with either pcoPG4 Δ NLS or pcoPG4 Δ CBS, in the context of the 4-component PFV vector system. Protein composition and the infectivity of the vector particles were determined.

We found wt-like cellular expression and wt-like amounts of released particles for both Gag mutants (Fig. 4A and B, lanes 4 to 8, anti-Gag), and the cellular expression levels of the viral Env and Pol were unaffected (data not shown). We found no reduction in Pol packaging for Gag Δ NLS and Gag Δ CBS (Fig. 4B, lanes 4 to 8, anti-PR/RT anti-IN), in contrast to reduced Pol levels in Δ GRII mutant particles.

Interestingly, relative infectivity compared to that of the wt was 27% for Gag Δ NLS as well as for Δ CBS. This represents an intermediate phenotype between the Gag wt (100%) and the Δ GRII mutant (5%).

Thus, Pol packaging is not affected by deletion of either the described CBS or NLS element of GRII. However, the infectious titer is reduced to similar extents by both deletions although less severely than for the Δ GRII mutant.

DISCUSSION

One of the most striking phenotypes that distinguish FVs from other retroviruses is a strong nuclear signal when infected cells are immunostained with sera from infected hosts (21). The majority of this nuclear staining results from the detection of PFV Gag protein that is localized to the nuclei of host cells during specific time points in the infection cycle (45). Although this has long been recognized as a hallmark of FV infection, the underlying mechanisms of PFV Gag nuclear translocation remain largely unknown. From previous studies, it seemed clear that GRII is essential for Gag nuclear localization. PFV

FIG. 6. Schematic illustration and intracellular localization of PFV Gag GRII box mutants. (A) Schematic outline of the generated PFV Gag GRII box mutant constructs established in the wild-type and C-terminal eYFP fusion context. The three Gag GR boxes are indicated as gray boxes. The p68/p71 PFV Gag cleavage site is shown as dashed line. Numbers indicate the amino acid position in Gag. Artificially introduced amino acids are indicated in italic type. The boxed amino acids indicate the wild-type sequence of GR II (45). CMV, cytomegalovirus virus promoter; SD, splice donor; SA, splice acceptor; pA, bovine growth hormone polyadenylation signal; CBS, chromatin-binding signal (52); NLS, nuclear localization signal (45, 57). (B) HeLa cells were transfected with expression plasmids for C-terminal eYFP-tagged Gag pcoPG4 CeYFP (row corresponding to wt in panel A), pcoPG4 CeYFP ΔGRII (ΔGRII), pcoPG4 CeYFP ΔNLS (ΔNLS), or pcoPG4 CeYFP ΔCBS (ΔCBS). The samples were fixed at 12 or 48 h p.t., stained with DAPI, covered in Mowiol, and analyzed by confocal laser scanning microscopy. Merge columns show merged images of the eYFP (green) and the DAPI (red) channel. eYFP (Gag) column shows the obtained signal in the eYFP channel. Scale bar: 10 μm.

Gag nuclear localization seems to be complex, because two motifs are described, both residing in GRII. These are a C-terminal NLS, which is proposed to mediate Gag transport to the nucleus, and an N-terminal CBS, which mediates interaction of Gag with H2A/H2B core histones (45, 52, 57). Similarly, multiple nuclear trafficking signals have also been described for HIV-1 Gag (5, 19), although these are disputed (10, 14). RSV (7) contains trafficking signals, which require tightly coordinated spatiotemporal control (18).

However, when we analyzed PFV Gag nuclear trafficking by live-cell imaging, we did not observe Gag trafficking to the nucleus in interphase cells. Gag was localized only in the cytoplasm and did not cross the intact nuclear membrane. Gag accumulated in the proximity of the nucleus, colocalizing with the MTOC (data not shown). Due to a maximum *z* resolution of approximately 500 nm, these accumulations sometimes appeared above or below the nucleus. However, cell fractionation and costaining with marker proteins (data not shown) confirmed their cytoplasmic localization. Only during mitosis, when the nuclear envelope breaks down, was Gag found bound to the host cell chromatin, and then the nuclear envelopes reformed around the Gag-chromatin complex, resulting in Gag being localized to the nuclei of the daughter cells.

We show that nuclear transport of Gag through the nuclear membrane is possible if a functional heterologous NLS is present. By fusion of a classical NLS, Gag can localize to the nucleus as early as 12 h p.t., which is never observed with wt Gag. The heterologous NLS is also functional when replacing

GRII, which proves the accessibility of this region for the nuclear localization machinery. However, artificial nuclear localization did not improve viral titers on G₁/S-phase-arrested cells. This is similar to data reported for spleen necrosis virus (SNV), which is known to deliver genes only into dividing cells (8). Furthermore, addition of a heterologous NLS to wild-type PFV Gag, resulting in import into interphase nuclei, reduced Pol incorporation, and viral infectivity. This suggests that simple delivery of PFV Gag to the nucleus is not sufficient for viral morphogenesis and Pol packaging, in particular, and raises the possibility that subtle differences in subcellular localization may play a role.

We found the CBS in GRII to be absolutely essential, whereas the described NLS is dispensable for nuclear localization. This contradicts earlier studies reporting that Gag localizes to the cell nucleus in an NLS-dependent manner (45, 57). Possibly, previous analyses of the overall cell population led to a different interpretation than dynamic single-cell analysis by live-cell microscopy.

Lo et al. recently reported SFVmac Gag nuclear import in growth-arrested cells to be dependent on the presence of integrase (31). They observed that, in the presence of an intact integrase, Gag, as part of the PIC, enters the nuclei of growth-arrested cells. Interestingly, in cycling cells they found no dependency of Gag nuclear localization on integrase. Using live-cell imaging, we never observed transport of Gag to the nucleus, regardless of the presence or absence of integrase. Possibly, this discrepancy reveals a minor difference between

the SFVmac Gag protein used by Lo et al. and the PFV Gag protein that we used.

Notably, GR11 is well conserved throughout all FV species, and the Gag proteins of PFV, SFV, equine FV (EFV), and bovine FV (BFV) were reported to transiently traffic through the nucleus (24, 30, 36, 53). Only for FFV Gag was a lack of nuclear localization reported (3). However, the overall conservation of this region suggests either an importance for Gag protein function or a specific sequence conservation of this region in the viral RNA genome.

Only very recently an NES was proposed for PFV Gag, at amino acid positions 95 to 112 (40). When we transiently expressed PFV Gag, most cells did not show a reappearance of Gag in the cytoplasm after initial localization of Gag to the nucleus during mitosis. For the cells for which we observed reaccumulation of Gag in the cytoplasm, it appeared to be due to *de novo* synthesis. Correspondingly, Gag did reaccumulate in the cytoplasm after being localized to the nucleus when it was constitutively expressed in an inducible manner from an integrated expression cassette, whereas no reaccumulation was observed when the cells were not continuously induced. Therefore, we did not observe evidence for Gag nuclear export in our limited, initial analysis.

Furthermore, Renault et al. describe a PFV Gag G110V mutant that was retained in the cell nuclei, with no viral particles being released from the cell (40). The different Gag SV40-NLS mutants used in our study, in particular Gag wt SV40-NLS 1 and Gag ΔGR11 SV40-NLS 1 mutant, show similar, complete redistributions of Gag to the cell nucleus. However, with both constructs we readily observed particle release in the 4-component PFV vector system. So, nuclear retention alone does not block PFV particle release.

What is the functional relevance of Gag nuclear localization? Deletion of the entire GR11 significantly reduces Pol packaging (33, 49), but by detailed analysis of GR11 we now reveal that Pol packaging is independent of nuclear localization. Furthermore, the Gag CBS was proposed to facilitate integration of the viral genome (52). Thus, the dependency of Gag on mitosis to enter the nucleus might be sufficient to explain why the FV genome is integrated only in dividing cells (31). However, our data suggest a more complicated interpretation, as artificial Gag nuclear localization does not increase PFV infectivity in nondividing cells.

For RSV Gag, nuclear trafficking plays a role in viral RNA export from the nucleus and is necessary for efficient incorporation of vRNA (16). Similarly, Renault et al. proposed a model in which FV Gag is required for nuclear export of the unspliced viral RNA (40). Controversially, Bodem et al. report that FV RNA is exported from the nucleus independent of viral proteins (2).

Potential functions of PFV Gag nuclear localization, therefore, remain to be defined. Our study significantly contributes to this question, because it clarifies the essential and necessary motifs present in PFV Gag. Furthermore, our data strongly imply that there is no functional NLS in PFV Gag mediating active nuclear import in interphase cells and that nuclear localization of PFV Gag is achieved only by tethering to accessible chromatin through the CBS motif during mitosis. This is a novel nuclear localization mechanism, further contributing to the distinction between FVs and the orthoretroviruses. It is

tempting to speculate that our data might also suggest a different functionality of Gag nuclear localization by passive chromatin tethering, compared to active, NLS-dependent nuclear localization.

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