Late maturation steps in the nucleus preceding pre-lytic active egress of progeny parvovirus

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Abstract

Although not well understood, growing evidence indicates that the non-enveloped parvovirus minute virus of mice (MVM) may actively egress from the nucleus before passive release through cell lysis. We have dissected the late maturation steps of the intranuclear progeny with the aim to confirm the existence of an active pre-lytic egress and to identify critical capsid rearrangements required to initiate the process. By performing anion-exchange chromatography (AEX), intranuclear progeny particles were separated by their net surface charges. Apart from empty capsids (EC), two distinct progenies of full capsids (FC) arose in the nuclei of infected cells. The earliest population of FC to appear was infectious but, similar to EC, could not be actively exported from the nucleus. A further maturation of this early population, involving N-VP2 exposure and phosphorylations of surface residues, gave rise to a second late population with nuclear egress potential. While the capsid surface phosphorylations were strictly associated to nuclear export capacity, mutational analysis revealed that the phosphoserine-rich N-VP2 was dispensable. A reverse situation was observed for the incoming particles, which were dephosphorylated in the endosomes acquiring the AEX profile of the early nuclear progeny without nuclear export potential. Our results confirm the existence of an active pre-lytic egress and reveal a phosphorylation-dephosphorylation cycle associated to nuclear import and export potential required for the replication of the karyophilic parvovirus.

Importance

In general, the process of egress of enveloped viruses is active and involves host cell membranes, however, release of non-enveloped viruses seems to rely more on cell lysis. At least for some non-enveloped viruses an active process before passive release by cell lysis has been described, although the mechanisms involved remain poorly understood. By using the non-enveloped model parvovirus minute virus of mice, we could confirm the existence of an active process of egress and further characterize the capsid maturation steps involved. Following DNA packaging in the nucleus, capsids required further modifications involving surface phosphorylations to acquire export potential. Those surface phosphorylations were removed from the entering capsids. This spatially and temporally controlled phosphorylation-dephosphorylation cycle would provide the nuclear import and export potential required for the infection of the non-enveloped karyophilic parvovirus.

Introduction

The egress of enveloped viruses is well characterized and involves budding trough host cell membranes (28, 58). The release of non-enveloped viruses is less well understood. In general, release of non-enveloped viruses is associated with cellular lysis, thus considered a passive process (10, 29, 53). However, there is accumulating data that an active egress precedes virus-induced cell lysis and subsequent passive release. For instance, bluetongue virus has been demonstrated to usurp the ESCRT machinery for egress by means of its L-domains (32, 59). Similarly, Hepatitis A virus release involves ESCRT-associated proteins (15). Furthermore, drug-induced stimulation of the autophagy pathway increased non-lytic spread of poliovirus and progeny virions were shown to accumulate unilaterally on the apical surface of polarized and productively infected epithelial cells (3, 54). Equally, simian vacuolating virus 40 and simian rotavirus were almost exclusively recovered from the apical culture fluid of polarized epithelial cells prior to cell lysis. Electron microscopy studies and specific inhibition of vesicular transport pathways indicate a vesicle-associated release of progeny virions (7, 17).

An active process of egress has also been suggested for parvoviruses (PV), a group of small, non-enveloped viruses (1, 2, 27). Autonomous rodent PVs, including minute virus of mice (MVM), display a T=1 icosahedral capsid containing a single-stranded DNA genome of about 5 kb (9). Due to their simplicity, PVs strongly depend on their host cell. Following entry, they are imported into the nucleus to profit from the replication machinery of the host for their own replication. Subsequently, assembly and genome packaging occur in the nucleus and give rise to the infectious progeny. Productive PV infection causes dramatic morphological and physiological changes of

their host cells, culminating in cell death (5, 16) and passive release of progeny virions. PV cytotoxicity is mainly mediated by the large non-structural protein NS1.

Besides passive egress by cell lysis, the existence of an active, pre-lytic egress for MVM has been suggested (1, 2, 27). Several important viral and cellular factors involved in PV egress have been identified. The highly stable interaction of the viral non-structural protein NS2 with Crm1 was suggested to play a role in egress (4, 39). Classical nuclear export signals (NES) exhibit low affinity for Crm1 to prevent the formation of stable Crm1/cargo complexes in the cytoplasm where RanGTP is absent (34). Surprisingly, the NES of NS2 belongs to the supraphysiological NES which tightly bind to Crm1 regardless of the presence of RanGTP. Therefore, NS2 competitively inhibits Crm1 function by sequestering endogenous nuclear export receptors (12). MVM mutants with disable Crm1 interaction were compromised in viral nuclear export and productive infection. (11, 31). The exact role of NS2 in virus egress was not elucidated and attempts to demonstrate an interaction of NS2 with viral capsid proteins were not successful. Since NS2 has multiple functions, abrogation of the tight NS2-Crm1 interaction might interfere with early functions during a productive infection which may indirectly affect progeny maturation and their export from the nucleus.

In human transformed cells, NS2 was dispensable for infection (35) and progeny export was not affected by treatment with the antifungal antibiotic leptomycin B, a drug which inhibits Crm1-dependent nuclear export. For these cells an alternative export mechanism was proposed involving the unordered N-terminus of VP2 (N-VP2) (27). Site-directed mutagenesis of the three distal serine residues at position 2, 6, and 10 of N-VP2 revealed an important role of these phosphorylations in the Crm1-independent nuclear export of MVM in permissive human transformed cells. When

the N-terminal phosphorylations were mutated, progeny virions showed an increased nuclear retention and displayed a small plaque phenotype, indicating the importance of those phosphorylations in viral spread (27).

Following nuclear export, it has been suggested that MVM is released actively through a vesicle-associated, gelsolin-dependent mechanism, involving major rearrangements of the cytoskeleton. Progeny virions were shown to co-localize with exocytic, endosomal, and lysosomal markers in immunofluorescent experiments. Cell fractionation experiments confirmed this observation by demonstrating a co-migration of viral particles with cytosolic vesicles (1). A co-operative cross-talk between actin and microtubule-dependent transport might be involved in MVM transport from the nucleus to the cell periphery (40, 46, 48).

The secretory pathway has been proposed as such a vesicle-dependent route for active egress of MVM. Progeny virions would become engulfed by COPII-vesicle formation in the perinuclear ER where they accumulated with dynamin. Accordingly, a dramatic retention of virions in the perinuclear area and inhibition of virion release into the medium was observed in cells lacking functional effectors of the secretory pathway (2). In addition, members of the ERM family, such as radixin and moesin, were shown to play a role in virus maturation and spreading capacity, as judged by their impact on MVM plaque morphology (37). Consequentially, dominant negative radixin or moesin mutants failed in wrapping progeny virions into transport vesicles, resulting in a marked reduction of egressed virions in the medium. Finally, active egress promotes cellular lysis as demonstrated by the prolonged viability of cells in which vesicular transport was either inhibited or by-passing the Golgi apparatus. Besides, the involvement of progeny particles in cytolysis was demonstrated by the pro-

longed survival of murine cells transduced with a viral vector deficient for the production of progeny virion particles (2).

Documentation of active egress by non-enveloped viruses requires accurate demonstration that no cell lysis occurred during the experiment. However, it is challenging to exclude the possibility that lysis of a few cells may passively release progeny virions, which could additionally contribute to uncontrolled second rounds of infection. Parvoviruses, particularly MVM, are highly robust and can persist as intact particles in the lysosomes of infected cells (25). Since the entry and the proposed egress route partially overlap in the dynamic endosomal pathway , the discrimination between incoming and progeny virions represents a major challenge.

The present investigation aims to confirm the existence of an active egress for MVM and to characterize the final capsid maturation steps leading to nuclear export and egress of MVM. Using anion-exchange chromatography (AEX) in combination with cell fractionation and quantitative PCR (qPCR) we demonstrate that two distinct populations of DNA containing progeny particles co-exist in the nucleus of infected murine cells. *De novo* synthesized capsids undergo a maturation step in the nucleus that involves surface phosphorylation(s) and exposure of N-VP2. Only mature virions were able to exit the nuclei and egress from the cells prior to cell lysis, confirming an active egress of parvovirus MVM.

In tissue culture, passive egress considerably contributes to viral spread. However, its importance in animal infection might be largely limited by clearance of virus-infected cells by components of the immune system.

Materials and Methods

Cells and viruses. A9 mouse fibroblasts (51) and NB324K cells (45), were routinely propagated under a minimal number of passages in DMEM supplemented with 5 % FCS at 37 °C in 5 % CO_2 atmosphere. Stocks of MVM were propagated on A9 cells. As soon as the cytopathic effect was complete, the supernatant was collected, pre-cleared from cell debris by low-speed centrifugation and the virus was pelleted through 20 % sucrose cushion. The virus pellet was washed and resuspended in PBS. Titers were determined by qPCR as DNA-containing particles per microliter. DNA-containing (full capsids; FC) and empty capsids (EC) were separated by CsCl gradient as previously described (42). CsCl was removed by size-exclusion chromatography through PD-10 desalting columns (GE Healthcare, Chalfont St Giles, UK) and when required, the capsids were concentrated in Amicon® centrifugal filter devices (Merck Millipore, Billerica, MA).

Antibodies, chemicals and enzymes. Rabbit anti-VPs (polyclonal against MVM structural proteins), rabbit anti-N-VP2 (polyclonal against the N-terminus of VP2), and mouse anti-capsid (monoclonal against intact capsids; clone B7) antibodies have been previously described (23, 27). Fluorescent-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and horseradish peroxidase-conjugated antibodies were purchased from DakoCytomation (Glostrup, DK). Bafilomycin A_1 (BafA1), chymotrypsin, and chymostatin were obtained from Sigma-Aldrich (St. Louis, MO) and reconstituted in ethanol at 0.1 mg/mL or in DMSO at 10 mM, respectively. To avoid enzymatic digestion or dephosphorylation during the processing of cell extracts, the lysis buffer was supplemented with protease inhibitors (Roche, Basel, CH); 1 mM sodium orthovanadate (Na_3VO_4), and 1 mM sodium fluoride (NaF) (Sigma-Aldrich).

Virus infection. A9 or NB324K cells (8×10^3 for qPCR or 3×10^6 for AEX) were infected with MVM (5000 DNA-containing particles per cell, corresponding to approximately 10 PFU/cell (50)) for 1 h at 4 °C for binding. Unbound virus was removed by washings and the cells were incubated at 37 °C to initiate infection. At progressive times post-internalization total cellular DNA was extracted for qPCR analysis or cells were fractionated and subjected to AEX.

Cell fractionation. A9 and NB324K cytoplasmic fractions were extracted in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail (Roche), pH 7.2 at 4 °C for 30 min. Following vortexing, intact nuclei and cell debris were removed by high-speed centrifugation at 4 °C. Isolation of nuclei was performed by using the Nuclei EZ Prep Nuclei Isolation Kit (Sigma-Aldrich) following the manufacturer's instructions. In order to obtain highly pure nuclear fractions, the isolated nuclei were further processed by centrifugation at 500 × g for 10 min through a sucrose gradient. The integrity of the isolated nuclei was examined by light microscopy after trypan blue staining. The purity of the nuclei and the absence of the outer nuclear membrane were evaluated with lamin A/C (inner membrane) and serca 2 ATPase (outer membrane) antibodies. Purified nuclei were lysed in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail (Roche), pH 7.2 at 4 °C for 30 min. Following vortexing, the nuclear lysate was passed 10 times through a 27 G needle and nuclear debris was removed by high-speed centrifugation at 4 °C.

Quantitative PCR (qPCR). Template DNA was extracted by using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, DE) following the manufacturer's guidelines. Amplification and real-time detection of PCR products was performed by using the CFX96™ Real-Time System with the iTaq™ Universal SYBR® Green Su-

permix (Biorad, Hercules, CA). Primers for MVM DNA amplification were: forward (5'-GACGCACAGAAAGAGAGTAACCAA-3'; nucleotides 231 to 254) and reverse (5'-CCAACCATCTGCTCCAGTAAACAT-3'; nucleotides 709 to 732). Specificity of the amplification was determined by melting curve analysis. As external standard, an infectious clone of MVM (30) was used in 10-fold serial dilutions.

Anion-exchange chromatography (AEX). The Mono Q HR 5/5 column (5 × 50 mm; Pharmacia, Uppsala, SW) was connected to the ÄKTApurifier 10/100 UPC-900 chromatography system operated by the UNICORN control software (GE Healthcare). The Mono Q column was equilibrated with five column volumes (CV) starting buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.2). Viruses (at least 10^8 virus particles) diluted in 1 mL starting buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) were applied to the Mono Q column trough a 2 mL injection loop, rinsed with six CV of starting buffer and eluted by a linear salt gradient (0-2 M NaCl) in 20 mM Tris-HCl, 1 mM EDTA, pH 7.2. The flow rate was constantly kept at 1.5 mL/min and salt concentration was monitored by electrical conductivity. Viruses in each fraction (185 µl) were quantified by qPCR.

Immunofluorescence microscopy. A9 cells (3×10^5) were seeded onto coverslips within 12-well plates. After 24 h, the cells were infected with 2'500 DNA-containing particles per cell, corresponding to approximately 5 PFU/cell (41), for 1 h at 4 °C. Subsequently, the cells were washed to remove unbound virus, and incubated at 37 °C. At different times, cells were washed and processed for immunofluorescence as previously described (21, 22) with fluorescent-conjugated secondary antibodies. Cells were mounted with Mowiol (Calbiochem, Billerica, MA) containing 30 mg/ml of DABCO (Sigma-Aldrich, St. Louis, MO) as an anti-fading agent and exam-

ined by laser scanning microscopy (LSM 510 Meta; 100× magnification objective, Carl Zeiss).

Immunoprecipitation. Viruses were incubated with specific antibodies in LoBind eppendorf tubes pre-blocked with PBSA (PBS containing 1 % BSA) for 1 h at 4 °C. Subsequently, 20 µL protein G agarose beads were added and the samples were further incubated overnight at 4 °C. The beads were washed with PBSA. To remove residual BSA an additional washing step was carried out with PBS.

Enzymatic reactions. All enzymatic reactions were performed in a 50 µL reaction volume. Viruses diluted in PBS (10^8 virus particles) were incubated for 1.5 h at 37°C with chymotrypsin (0.5 mg/mL) and the reaction was stopped by adding 100 µM chymostatin. Lambda phosphatase treatment (40000 U/mL) was performed in 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MnCl_2$, 5 mM DTT, pH 7.8 for 3 h at 37 °C. Phosphatase was inactivated by adding 1 mM Na_3VO_4 and 1 mM NaF. Free DNA was digested using DNase I (50 U) at 37 °C for 1.5 h. DNase I was inhibited by incubation at 75 °C for 15 min.

Results

Two distinct populations of progeny DNA-containing particles are detectable in the nucleus of MVM infected cells. Progeny viral particles released in the supernatant of infected cell cultures were collected 8 days post-infection (dpi), when cytopathic effect was complete. Cell debris was excluded by centrifugation. The collected viral capsids were analyzed by anion-exchange chromatography (AEX), which can separate particles based on their net surface charges, followed by quantitative PCR (qPCR). Two distinct virus populations of DNA-containing particles (full capsids; FC) were separated and their relative amount quantified by qPCR (Fig. 1A). A second AEX-qPCR analysis was performed in parallel, which included both medium and cell lysate obtained by freeze and thaw cycles to release the remaining intracellular viruses. By including these additional intracellular viruses, the same two populations were detected but their proportion was different. The more positively charged population (referred to as FC progeny 1; FC-P_1) was predominantly associated with cells, thus it increased when more intracellular viruses were included. In contrast, the more negatively charged population (referred to as FC progeny 2; FC-P_2) was the predominant population in the supernatant when most of the intracellular viruses were excluded.

In order to verify the integrity of the two DNA-containing viral populations, we collected supernatant and intracellular viruses and subjected them to nucleolytic digestion. As shown in Fig. 1B, both virus populations were resistant to nuclease digestion and their AEX profile did not change, indicating that both particle types represent assembled DNA-containing particles.

MVM capsid assembly occurs in the nucleus. It was therefore of interest to verify the presence of both virus populations in the nucleus early at the onset of as-

sembly and packaging. Viruses were collected from isolated nuclei of infected murine fibroblasts early after infection and subjected to AEX-qPCR. As shown in Fig. 1C, by 18 hpi both DNA-containing viral populations co-existed in the cell nucleus in similar amounts.

Besides DNA-containing capsids, MVM infection results in the accumulation of empty capsids (EC), which represent assembled capsid precursors that have not yet packaged viral genomes (33). To verify their AEX profile, EC precursors were purified by CsCl, subjected to AEX and visualized by dot blot using an antibody against assembled capsids (Mab B7) (23). As illustrated in Fig. 1D, EC had an AEX profile resembling that of the FC-P_1 population. The fact that FC-P_1 particles are predominantly cell-associated and have a similar AEX profile to that of the EC precursors would suggest that they represent immature particles without egress potential, whereas FC-P_2 would represent particles displaying a further maturation step enabling active release.

FC-P_1 and FC-P_2 are infectious and differ in N-VP2 conformation. In order to further characterize the two FC populations, we separated them by AEX, pooled the fractions corresponding to each population and performed a second AEX. In Fig. 2A the chromatograms of purified FC-P_1 and FC-P_2 are shown. The purified viral populations were used to investigate their capacity to initiate the infection in A9 cells. As demonstrated in Fig. 2B, both virus populations were able to reach the nucleus and their genomes were replicated without significant differences.

In MVM virions the N-terminal region of the VP2 (N-VP2) occupies an external positon in the capsid, however, during entry N-VP2 is cleaved by endosomal proteases to render a shorter protein named VP3 (52, 56). The function of N-VP2 cleavage is not fully understood, but it is required to allow the exposure of the N-terminal

region of VP1 (N-VP1) (8, 13), which harbors important functional motifs essential for the infection (55), particularly endosomal escape (14) and nuclear targeting (22). We analyzed the surface conformation of N-VP2 in the two populations of FC by immunoprecipitation with a specific antibody raised against this region (27). As demonstrated in Fig. 2C, N-VP2 occupies a surface position in FC-P_2 but is predominantly sequestered in FC-P_1. Accordingly, FC-P_1 resembles to EC also in the sequestered N-VP2 conformation. In contrast to EC, exposure of FC-P_1 to temperature (50 °C) or to acidic conditions (pH 4.5) resulted in a significant externalization of N-VP2 (Fig. 2D). Chymotrypsin (CHT) has been previously demonstrated to mimic the *in vivo* cleavage of N-VP2. EC do not expose N-VP2 on the capsid surface and thus they cannot be cleaved. The AEX-purified capsid populations were subjected to proteolytic digestion by CHT under neutral and acidic conditions. As shown in Figure 2E, FC-P_2 particles were completely processed under all tested conditions. In contrast, the N-VP2 of FC-P_1 was only marginally accessible to CHT under neutral conditions and required acidification to improve the cleavage rate, confirming its predominant internal conformation. The substantial amount of VP2 that remained unprocessed originates from EC, which elute in the same AEX fractions as the FC-P_1.

When incubated with A9 cells, both virion progenies bound similarly to cells as shown by immunofluorescence. The N-VP2 from FC-P_2 was detectable on the surface of the cells and was fully processed by 4 hpi. As expected, the N-VP2 from FC-P_1 was not visible at binding but became exposed after internalization and a proportion remained detectable for several hpi (Fig. 2F), indicating a slower or less efficient VP2 to VP3 processing.

We next investigate whether the sequestered N-VP2 conformation in FC-P_1 is responsible for its distinct AEX profile. To this end, FC-P_1 virions were exposed to

acidic conditions to provoke the externalization of N-VP2 (as shown in Figure 2D) and analyzed by AEX-qPCR. The results showed that despite the externalization of N-VP2, the AEX profile remained unchanged. Accordingly, the distinct N-VP2 conformation is not responsible for the different AEX profile. The results also suggest that packaging does not directly result in N-VP2 externalization and further emphasize similarities between the FC-P_1 progeny virions and the EC precursors.

The surface phosphorylation status of the capsid is a key determinant of the different AEX profile. In order to further examine biochemical and structural differences between the two FC populations, both capsid species were isolated from the nuclei of infected A9 cells, treated with lambda phosphatase and subsequently analyzed by AEX-qPCR. When dephosphorylated by treatment with lambda phosphatase, FC-P_2 changed its AEX profile to that of FC-P_1, which remained unchanged (Fig. 3A). This result suggests that the differences between the two FC populations are due to a distinct surface phosphorylation status, other than the distal phosphoserines in N-VP2 (26). To confirm this, we used a MVM mutant, in which all distal serine residues on N-VP2 were substituted by glycine (referred to as 5SG). Similar to the wild-type (WT), the 5SG mutant generated also FC-P_1 and FC-P_2 particles (Fig. 3B) and pre-treatment with lambda phosphatase generated mostly FC-P_1 particles (Fig. 3C) These results confirm that additional phosphorylation(s), other than the distal phosphoserines in N-VP2, are present exclusively in the FC-P_2 population and are responsible for their specific AEX profile.

Only FC-P_2 has nuclear export potential and can actively egress from the infected host cell. The subcellular distribution of the two full capsid progenies was examined. A9 cells were infected with MVM and at progressive times pi, progeny viruses were collected from nuclear, cytoplasmic and supernatant fractions and sub-

jected to AEX-qPCR. While in the nucleus both FC populations accumulated with similar kinetics, in the cytoplasm and in the supernatant, the accumulation of FC-P_2 preceded that of FC-P_1 (Fig. 4 A-C). The FC-P_2 egress started largely before the onset of cell lysis, which occurred from 30 hpi, as judged from the trypan blue exclusion assay (Fig. 4E) and resulted in the appearance of FC-P_1 in the cytoplasmic and supernatant fractions. The compartment-dependent segregation of the two full capsid progenies at increasing times pi reveals the existence of an active mechanism of nuclear export and egress involving exclusively FC-P_2 particles and preceding the passive release of FC-P_1 and EC through late virus-induced cell lysis.

FC-P_1 is the precursor of FC-P_2. During infection in the presence of neuraminidase and anti-capsid antibody to prevent reinfections, FC-P_1 was the first population to appear in the nucleus of murine A9 and human transformed NB324K cells. While in NB324K cells FC-P_1 disappeared progressively to give rise to FC-P_2, in A9 cells this transfer was less efficient, leading to the accumulation of both populations (Fig. 4A and Fig. 5A). In order to further confirm FC-P_1 as precursor of FC-P_2, NB324K cells were transfected in the presence of neuraminidase and anti-capsid antibody. FC-P_1 and FC-P_2 progeny virions were quantitatively analyzed by AEX-qPCR at 24 and 48 hpt when no significant *de novo* production or degradation of viral progeny particles was observed (Fig. 5B). As shown in Fig. 5C, FC-P_1 was the predominant virus population 24 hpt, representing approximately two third of the total progeny virions. However, 48 hpt the total amount of FC-P_1 virions significantly declined representing only one third of the whole virus progeny and giving rise to a significant increase in the amount of FC-P_2 DNA-containing particles. Collectively, these results indicate that FC-P_1 particles are the precursors of FC-P_2 virions. The matura-

388 tion of FC-P_1 into FC-P_2, which involves surface phosphorylations, would be more
389 efficient in the human transformed cells than in the A9 murine fibroblasts.

390 **The phosphoserine-rich N-VP2 is dispensable for active egress.** In sharp
391 contrast to FC-P_1 and empty particles, FC-P_2 capsids are exported from the nucleus
392 and subsequently released from the host cell prior to cell lysis. Distinct from EC or
393 from FC-P_1, N-VP2 of FC-P_2 is external and the capsids have additional capsid sur-
394 face phosphorylations. These features represent a late maturation step and might
395 confer the nuclear export potential to FC-P_2. N-VP2 is heavily phosphorylated at ser-
396 ine residues, which have been previously suggested to assist nuclear export in a cell-
397 specific manner. Mutants lacking N-VP2 distal phosphoserines were deficient in nu-
398 clear export and egress in NB324K cells, but not in A9 murine cells (27). We used a
399 mutant in which the four distal serine phosphorylations on the N-VP2 terminus and an
400 additional serine in the poly-glycine region, were substituted by glycine (referred to as
401 5SG). Additionally, we used a MVM mutant containing a bulky phenylalanine residue
402 at position 33 within the flexible poly-glycine stretch (referred to as G33F) (Fig. 6A).
403 Due to this substitution the mutant progeny particles were unable to externalize N-
404 VP2 following DNA packaging. Accordingly, transfection with this mutant generated
405 DNA-containing particles that were not infectious due to failure to expose and pro-
406 cess N-VP2 and to externalize VP1u during entry (6). Upon transfection, DNA-
407 containing progeny particles of 5SG and G33F progressively accumulated in the cell
408 culture medium to similar quantities and kinetics as the WT virions (Fig. 6B). AEX
409 analysis revealed that the G33F, as well as the 5SG progeny, consisted of both FC-
410 P_1 and FC-P_2 particles (data not shown), further substantiating that N-VP2 and/or its
411 distal phosphorylations are not responsible for the two distinct AEX profiles of FC-P_1
412 and FC-P_2. Transfection results in an increased cell lysis due to cell damage and

413 therefore, it is expected to increase passive release. To examine whether the ob-
414 served extracellular accumulation of G33F, 5SG, and WT progeny particles resulted
415 mostly from active egress, we analyzed the intra- and extracellular FC-P_1/FC-P_2 rati-
416 os 24 hpt. At this time, the FC-P_1 population exceeded that of FC-P_2 in the nucleus,
417 however, in the extracellular milieu the ratio was inversed (Fig. 5C and 6C). The in-
418 verted ratios can only be explained by the existence of an active egress of FC-P_2 par-
419 ticles, despite the presence of an increased passive release due to transfection-
420 mediated cell damage. These results emphasize that N-VP2 sequences and its distal
421 phosphorylations do not play a direct role in the nuclear export and egress of MVM.

422 **The nuclear FC-P_2 particles represent the ultimate maturation step of**
423 **MVM in terms of egress potential and infectivity.** We next examined whether the
424 nuclear FC-P_2 population represents the final maturation step in MVM morphogene-
425 sis or whether further maturation steps occur during the process of egress required
426 for infectivity. To this end, nuclear and extracellular FC-P_2 particles were purified by
427 AEX and their infectivity quantitatively compared. Apart from having the same AEX
428 profile, both particles were equally infectious (Fig. 7A). Therefore, virus egress does
429 not involve further maturation steps of the progeny required for infection.

430 **During entry, FC-P_2 particles are dephosphorylated acquiring the AEX**
431 **profile of FC-P_1.** In the nucleus, FC-P_1 particles maturate through surface phosphor-
432 ylations to generate FC-P_2, which are particles with nuclear export potential. During
433 entry a reverse situation was observed, FC-P_2 particles were processed to generate
434 FC-P_1-like particles (Fig. 8). The N-VP2 of incoming FC-P_2 became cleaved by endo-
435 somal proteases (Fig. 2F). However, as previously shown in Figure 2G and 3B, the
436 presence or absence of N-VP2 with its distal phosphoserines has no influence in the
437 AEX profile. As already demonstrated *in vitro* (Fig. 3A), the different AEX profiles re-

sults from the presence of capsid surface phosphorylations. Accordingly, these critical surface phosphorylations are removed from the incoming FC-P_2 early during entry by endosomal acid phosphatases. In order to confirm the involvement of acid phosphatases in the processing of incoming particles, bafilomycin A1 (BafA1), which raises the endosomal pH and inhibits acid phosphatases, was applied to A9 cells. As shown in Fig. 8, BafA1 totally abrogated the dephosphorylation of the incoming FC-P_2 population.

Discussion

The active egress of enveloped viruses is well documented and involves budding through host cell membranes. The egress of non-enveloped viruses is generally thought to be the result of a lytic burst occurring at the end of the infection. However, there is growing evidence that it may not be a mere consequence of the passive virus release induced by cell lysis but involves a pre-lytic active transport of the progeny virions (2, 3, 7, 15). The proof of an active pre-lytic egress for non-enveloped viruses is challenging since the lysis of a few cells can passively release mature virions complicating the discrimination between active and passive release. Therefore, it is not sufficient to detect progeny virions in the culture media prior to significant cell lysis. In addition, the identification of an active mechanism is necessary. By using anion-exchange chromatography (AEX) and cell fractionation, we confirmed the existence of an active egress for the model parvovirus minute virus of mice (MVM) prior to passive release by cell lysis. Additionally, we identified late capsid maturation steps occurring in the cell nucleus preceding nuclear export.

The current model of MVM morphogenesis and egress suggests that EC precursors are first assembled in the nucleus and subsequently filled with the viral ssDNA to generate FC progeny (19). As a consequence of packaging, the phosphoserine-rich N-VP2 becomes exposed outside of the shell through the fivefold axis of symmetry (9, 52). The exposed N-VP2 has been suggested to mediate the export of the FC progeny out of the nucleus (27), followed by virus egress, which was proposed to occur by vesicular transport through the endoplasmic reticulum and Golgi (2).

By using AEX, proteins can be separated based on their net surface charges. We performed AEX to separate and characterize parvovirus progeny particles dis-

playing different protein surface configurations. Apart from EC precursors, the AEX profile of intranuclear MVM progeny revealed not one but two well-defined DNA-containing progeny populations, here named FC-P_1 and FC-P_2. FC-P_1 progeny shares many characteristics with the EC precursors. They appeared early, had a similar surface phosphorylation pattern, N-VP2 was essentially inaccessible and the particles were unable to be exported from the nucleus. FC-P_2 virions appeared later, featured additional surface phosphorylations, N-VP2 was exposed and they showed nuclear export potential. FC-P_1 would represent a previously unrecognized stage in the MVM morphogenesis, intermediate between EC precursors and the late FC-P_2 virions. The nuclear export competent FC-P_2 virions represent the fully mature infectious progeny. The infectivity of FC-P_2 progeny isolated from the nuclei (pre-egress) or actively released from the cells (post-egress) was the same (Fig. 7). Hence, during active egress no further maturation steps were required to acquire full infectivity.

The internal conformation of N-VP2 in FC-P_1 indicates that, in contrast to the general assumption, DNA packaging alone is not sufficient to trigger the externalization of N-VP2. Interestingly, the distinct N-VP2 conformation between the two FC populations was not responsible for their different AEX profile. Heat treatment or incubation at low pH externalized the N-VP2 termini of FC-P_1 but did not change its AEX profile.

The N-VP2 termini, particularly their distal serine phosphorylations, have been previously suggested to play a crucial role in the nuclear export of *de novo* synthesized virion progeny (27). This data is in line with our findings which demonstrate that following packaging, FC-P_1 particles with internal N-VP2 did not have nuclear export capacity. In order to challenge a possible involvement of N-VP2 and its prominent

distal phosphorylations in the export of the late progeny FC-P_2 population, we used two mutants. The first mutant, referred to as 5SG, lacks the five most distal serine phosphorylations within the N-VP2 termini. The second mutant, referred to as G33F, is unable to externalize the N-VP2 sequence on the surface of the capsid due to the insertion of a bulky phenylalanine residue at position 33 of its poly-glycine stretch within the VP2 protein sequence (Fig. 6A). Confirming our previous observations, removal of the distal serine phosphorylations of N-VP2 or prevention of its externalization had no influence in the different AEX profiles, which was exclusively defined by additional surface phosphorylations (Fig. 3B and 3C). Following transfection in NB324K cells, both mutants were able to generate the early FC-P_1 and the late FC-P_2 progeny populations in the nucleus and accumulated in the culture media with similar kinetics and quantities as observed for the WT (Fig. 3B, 6B and 6C). These results confirm that the N-VP2 termini and their distal serine phosphorylations are not key players in virus egress. Parvoviruses display a high mutation rate comparable to RNA viruses (43, 44). Accordingly, genetic substitutions that interfere with crucial stages of the viral life cycle result in reversions after only a few rounds of infections. The distal S/G substitutions in N-VP2 were highly stable and no genetic reversions were observed following several passages (data not shown). In agreement with our findings, a deletion of seven amino acids within the sequence of N-VP2 with the intention to disturb its function did not affect egress of progeny particles. However, this truncation caused a retarded entry, leading to a delayed progeny egress. Additionally, this mutant showed a lower cytotoxic effect (56). MVM infection is reported to induce dramatic changes to the cytoskeleton of the host cell resulting in cell rounding and detachment, culminating in lysis and passive progeny virus release (36). The 5SG mutant showed a similar retardation of infection, accompanied by significantly reduced morphological alterations to the cytoskeleton of infected murine cells. Dis-

535 similar to wt infections, A9 cells infected with 5SG virions remained intact and dis-
536 played the characteristic fibroblastic phenotype as late as 40 hpi.

537 Apart from the N-VP2 conformation, the surface phosphorylation pattern is the
538 second prominent difference between FC-P_1 and FC-P_2 (Fig. 3). Therefore, it is
539 tempting to speculate that the acquirement of additional surface phosphorylation(s)
540 confers nuclear export potential to the late progeny population. There are alternative
541 nuclear export routes that function in higher eukaryotes independently of the
542 Crm1/exportin1 pathway involving the prototypic leucine-rich NES (20, 49); reviewed
543 in reference (24). These export mechanisms are predominantly regulated by protein
544 phosphorylation (18, 38). Accordingly, the additional capsid surface phosphorylations
545 in FC-P_2 may explain their nuclear export potential. In line with this notion, these sur-
546 face phosphorylations were efficiently removed by acidic endosomal phosphatases
547 during entry of incoming virions, resulting in a complete reversion to FC-P_1 particles
548 (Fig. 8). Together with N-VP2 cleavage and N-VP1 externalization, the dephosphory-
549 lation of surface residues would represent a novel processing step during parvovirus
550 cell entry which could be critical to stabilize incoming virions inside the nucleus of
551 infected cells. In line with this concept, it has been previously shown that the endo-
552 cytic route is required for nuclear targeting of CPV and AAV. Particles microinjected
553 into the cytoplasm to bypass the endocytic route failed to target the nucleus, even
554 when pretreated under acidic conditions (47, 57).

555 Heat or acidic treatment did not change the AEX profile of either progeny pop-
556 ulation, even though causing major structural transitions. Therefore, the phosphoryla-
557 tion of FC-P_1 to generate FC-P_2 particles should be mediated by resident nuclear
558 kinase(s) rather than by structural rearrangements exposing phosphorylated resi-
559 dues. The efficiency to achieve this late phosphorylation step was cell type depend-

ent, being more efficient in the human transformed NB324K than in the murine A9 cells. The more efficient phosphorylation in is in agreement with previous studies reporting lower overall capsid phosphorylation levels in murine A9 cells compared to the transformed human cells (26).

The requirement of NS2 in progeny egress has already been demonstrated to be indispensable for murine A9 cells but it is not a prerequisite in transformed NB324K cells. NS2 harbors a supraphysiological NES and tightly interacts with Crm1. Prevention of the NS2-Crm1 interaction has previously been demonstrated to impede nuclear export of progeny virions in restrictive mouse fibroblasts (11, 31). These results explain the previously observed cell type specific inhibition of nuclear export by leptomycin B (LMB) (27). However, NS2-Crm1 interaction is not required for the late nuclear maturation of the virion progeny. A mutant containing amino acid substitutions within the consensus NES sequence produced WT levels of FC-P_2 progeny but nuclear export of the fully mature progeny was blocked (data not shown). Despite extensive attempts, demonstration of a direct or indirect interaction between N-VP2 or other capsid regions and Crm1 failed. Therefore, the dependence of progeny egress on the Crm1 export pathway may rather be indirect via the supraphysiological interaction between NS2 and Crm1 (12).

The identified spatially and temporally controlled changes in capsid surface phosphorylation would provide nuclear import and export potential required to complete the life cycle of the karyophilic virus. Further studies are required to identify the corresponding phosphorylations on the capsid surface and to demonstrate their specific role in the active egress of the non-enveloped parvovirus.

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Legends for figures

Figure 1: Isolation of two distinct populations of *de novo* DNA-containing particles. (A) Viruses (10^{10} DNA-containing particles) were collected 8 dpi from the culture media of infected A9 monolayers (SN). Intracellular particles were released by repeated freezing and thawing cycles (SN + Cells). Anion-exchange chromatography (AEX) was performed and fractions were collected. DNA-containing particles in each fraction were quantified by qPCR. (B) Prior to AEX-qPCR, viruses were treated with 50 U DNase I. (C) A9 cells (3×10^6) were infected at a MOI of 5'000 DNA-containing particles per cell for 1 h at 4 °C, followed by washing to remove unbound virus. The cells were further incubated at 37 °C for 18 h. Nuclei were purified and the nuclear progeny was subjected to AEX-qPCR analysis. To avoid re-infections, neuraminidase and α-capsid mAb were added to the cells . (D) EC isolated from infected A9 cells were subjected to AEX followed by dot blot using the α-capsid mAb for detection.

Figure 2: FC-P_1 and FC-P_2 are infectious but differ in their N-VP2 conformation. (A) Fractions enriched in FC-P_1 or FC-P_2 (fractions 10-12 and 14-17, respectively, see Fig. 1A) were pooled, dialyzed in TE-buffer pH 8 and re-subjected to AEX. DNA-containing particles in each fraction were quantified by qPCR. (B) A9 cells (8×10^3) were infected with purified FC-P_1 or FC-P_2 particles at a MOI of 2500 DNA-containing particles per cell for 1 h at 4 °C, followed by washing to remove unbound virus. The cells were further incubated at 37 °C for 40 min or 22 h. Total DNA was extracted and quantified as described in Materials and Methods. (C) Immunoprecipitation of 10^8 FC-P_1 or FC-P_2 particles with a B7 α-capsid mAb (total) or a rabbit α-N-VP2 pAb. Specificity of the antibodies was confirmed using unspecific rabbit IgG. (D) FC-P_1 particles (10^8) were incubated at 50 °C or at pH 4.5. Immunoprecipitation was performed

as explained above. (E) Purified FC-P_1 or FC-P_2 particles (10^8) were incubated at pH 7, 6, or 5 and subjected to CHT treatment (+) or not (-). Proteolytic N-VP2 processing was analyzed by 10 % SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, the blot was probed with a rabbit α-VP pAb, followed by a horseradish peroxidase-conjugated secondary antibody. (F) A9 cells (3×10^5) were infected with purified FC-P_1 or FC-P_2 as indicated above. At different intervals pi the proteolytic processing of N-VP2 was examined by immunofluorescence with B7 α-capsid mAb (green) and α-N-VP2 pAb (red). (G) Nuclear progeny (10^8 DNA-containing particles, see Fig. 1C) was treated with CHT (0.5 mg/mL) for 1.5 h at 37 °C and subjected to AEX-qPCR analysis. (H) Purified FC-P_1 particles (10^{10}) were treated at pH 7 or pH 4.5 followed by dilution in TE-buffer pH 8 and AEX analysis. Viral DNA in eluted fractions was quantified by qPCR.

Figure 3: The surface phosphorylation status determines the AEX profiles of FC-P_1 and FC-P_2. (A) Nuclear virus progeny (10^{10} DNA-containing particles) was treated with lambda phosphatase (40000 U/mL) for 3 h at 37 °C prior to AEX-qPCR analysis. (B) A9 cells (3×10^6) were infected with 5SG mutant viruses as previously described. Nuclear progeny virions (10^{10} DNA-containing particles) were analyzed by AEX-qPCR analysis. (C) An identical amount of nuclear 5SG progeny virions were treated with lambda phosphatase as outlined above and subjected to AEX-qPCR analysis.

Figure 4: FC-P_2 progeny actively egresses from the infected host cell. A9 cells (3×10^6) were infected with 5000 DNA-containing particles per cell at 4 °C. Following washing to remove unbound viruses the cells were incubated at 37 °C in the pres-

ence of neuraminidase and B7 for the indicated times. Then, cells were fractionated as explained in Materials and Methods and subjected to AEX-qPCR analysis. Relative amounts of FC-P_1 and FC-P_2 virions were calculated and plotted. (A) Progeny in the nuclei of infected A9 cells. (B) Progeny in the cytoplasm of infected A9 cells. (C) Progeny in the media of infected A9 cells. (D) Phase contrast pictures of the infected cells were taken using a Zeiss Axiovert 35 microscope with a 20× magnification objective. Cell viability was accessed via trypan blue exclusion using the TC10^{TM} automated cell counter (BioRad). The average of three independent measurements is indicated.

Figure 5: Dynamics of FC-P_1 and FC-P_2 in infection and transfection. (A) A9 cells (3×10^6) were infected with 5000 DNA-containing particles per cell at 4 °C. Following washing to remove unbound viruses the cells were incubated at 37 °C in the presence of neuraminidase and B7 for the indicated times. Nuclei isolation and AEX-qPCR analysis were performed at the indicated time points post-infection as specified in Materials and Methods. (B) NB cells (10^6) were transfected in the presence of neuraminidase and B7 and intracellular virus was immunoprecipitated with B7 mAb and quantified at the indicated time points post-transfection. (C) NB cells were transfected as explained above. AEX-qPCR analysis was performed at the indicated time-points.

Figure 6: The phosphoserine-rich N-VP2 is dispensable for active egress. (A) Schematic representation illustrating the introduced mutations used for this transfection experiments. (B) NB cells (10^6) were transfected in the presence of neuraminidase and B7. Egressed viruses in the media were quantified following DNaseI treat-

ment. (C) AEX-qPCR analysis of intracellular and released virions was performed 24 hpt and FC-P_1 to FC-P_2 ratios were calculated.

Figure 7: Influence of active egress in infectivity. FC-P_2 particles were purified by AEX from the nuclei of infected A9 cells and from the culture media supernatant. A9 cells (8×10^3) were infected at 4 °C for 1h. Following removal of the unbound virus the cells were incubated at 37 °C for the indicated times. Intracellular DNA was extracted and viral genome copies were quantified.

Figure 8: Investigation of the effect of acidic phosphatases on surface phosphorylations during entry. A9 mouse fibroblasts (3×10^6) were infected with 5000 DNA-containing FC-P_2 particles per cell at 4 °C. Following removal of unbound viruses, cells were incubated at 37 °C for the indicated times. In order to inhibit acidic phosphatases, 150 nM BafA1 was added 15 min prior to virus internalization at 37 °C.

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