Discussion:

The study of viral egress is of interest since it directly affects the transmission and proliferation of virus progeny trough the host’s tissue. The egress of enveloped viruses is well documented. At the end of their replication cycle they form vesicles at the host’s plasma membrane and egress via budding. The egress of non-enveloped viruses is poorly understood. It has long been thought that the egress of non-enveloped viruses is the result of a lytic burst occurring at the end of a virus infection. However, there is growing evidence that egress of non-enveloped viruses is not a mere consequence of the passive virus release induced by cell lysis but involves a prelytic active vesicular transport of the progeny virions (Nüesch..).

In the case of enveloped viruses, actively egressing progeny acquires a host cell membrane derived envelope and thus it is simply distinguishable from passively released viruses lacking such envelope. The documentation of an active prelytic egress of non-enveloped viruses is complex since the lysis of only few cells can passively release virions that might be interpreted as actively egressing progeny. Therefore, it is of importance to not only demonstrate the presence of virions in the culture media prior to cytolysis but to directly show an active retrograde transport of virus progeny. By using anion-exchange chromatography (AEX) we were able to identify a late nuclear maturation step leading to a segregation of two DNA-containing virion populations. Only one population was able to actively egress the cell prior to cell lysis, confirming an active egress for MVM, a commonly accepted model parvovirus.

The two DNA-containing progeny populations differed in their net surface charges in consequence of distinct phosphorylation states resulting in distinctive AEX profiles. Interestingly, ECs displayed an identical AEX profile as the FC-P1 population, indicating a similar phosphorylation pattern on their capsid surface. Significantly, both ECs and FC-P1 were incapable of nuclear export. In contrast, the nuclear export competent FC-P2 virions featured a more negatively charged capsid surface caused by additional phosphorylations. Moreover, the N-VP2 conformation represented a major structural difference between FC-P1 and FC-P2. While externalized in FC-P2, the N-VP2 termini of FC-P1 were not accessible to antibodies or proteolytic digestion as previously observed for ECs (ref). This observation encouraged us to investigate a potential involvement of N-VP2 in the nuclear export of MVM.

The N-VP2 termini, particularly their distal phosphorylations, have been previously reported to play a crucial role in the nuclear export of *de novo* synthesized virion progeny (4SG, Almendral). This data is in line with our findings that nuclear export capacity was observed only for the N-VP2 exposing FC-P2 virions. In order to challenge a possible involvement of N-VP2 and its prominent distal phosphorylations in the export of FC-P2 virions, we used two genomic mutants. The first mutant, referred to as 5SG, lacks the five most distal serine phosphorylations within the N-VP2 termini. The second mutant, called 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 VP2 protein sequence. Our results revealed that both the removal of the distal serine phosphorylations of N-VP2 and the prevention of its externalization by site-directed mutagenesis preclude N-VP2 from being the determinant for the different AEX profiles. Both mutants were still able to produce FC-P1 as well as FC-P2 progeny virions, although having altered N-VP2 configurations. These findings point to other surface phosphorylations distinct from the distal N-VP2 serine phosphorylations determining the two characteristic AEX profiles.

Unexpectedly, the aforementioned mutants were able to accumulate in the culture media to similar quantities as observed for the WT. Analysis of the intra- and extracellular AEX profiles following transfection in transformed human fibroblasts revealed that, equally to the WT virions, mainly FC-P2 particles were accumulating in the media, indicating an active prelytic egress also for the mutant virions. These results appear to be contradictory to a previous report where the N-VP2 termini and their distal serine phosphorylations have been shown to drive the capsid out of the host’s nucleus (4SG, Almendral). However, importance of the distal phosphorylations of N-VP2 for nuclear export has been only demonstrated for transformed human fibroblasts. In murine fibroblasts but not in transformed human fibroblasts, progeny egress was sensitive to inhibition of Crm1 by LMB and required the infection process itself. Despite extensive attempts, demonstration of a direct or indirect interaction between N-VP2 or other capsid regions and Crm1 failed. The dependence of progeny egress on the Crm1 export pathway may rather be indirect via the supraphysiological interaction between NS2 and Crm1 (ref). The requirement for NS2 in progeny egress has already been demonstrated to be indispensable for murine cells (Pintel, Salomé), explaining the cell type specific inhibition of nuclear export by LMB treatment (Almendral, 4SG). Moreover, a 7 aa deletion in the trypsin sensitive RVER region within the sequence of N-VP2 did not affect egress of progeny particles. The only limitations reported for this truncation were a slight impairment for binding and nuclear targeting and thus a delayed time course of infection (Pintel, own observations).

Apart from the N-VP2 conformation, the surface phosphorylation pattern is the second prominent difference between FC-P1 and FC-P2. The surface phosphorylations responsible for the characteristic AEX profiles for FC-P1 and FC-P2 changed during a productive infection. Following pre-assembly of ECs, FC-P1 was the first progeny virion population to occur in the nucleus and was transferred into FC-P2 particles in corresponding stoichiometric amounts. FC-P2 was the only population able to be exported from the nucleus. Therefore, it is tempting to speculate that the acquirement of additional surface phosphorylation(s) confers a nuclear export potential to the FC-P2 population. Moreover, these surface phosphorylations providing a putative nuclear export capacity to FC-P2 particles were removed by acidic endosomal phosphatases during endocytosis of incoming FC-P2 virions, resulting in a complete reversion towards FC-P1 phenotype. These findings are fully compatible with respect to the life cycle of karyophilic viruses. Additionally, it has been previously reported that the endocytic route is required for nuclear targeting of CPV and AAV (ref). The dephosphorylation of surface residues may represent a fourth requirement besides N-VP2 cleavage, N-VP1 externalization and DNA exposure to being achieved during endosomal processing prior to nuclear targeting.

Outlook: identification of the surface phosphorylations and study their direct or indirect implications in the egress of non-enveloped viruses.

GTP (56), though other types of NES can also

bind CRM1 (3, 20). This rapid CRM1/exportin 1 pathway of

nuclear export can be inhibited by the antifungal antibiotic

leptomycin B (LMB) (28). In addition, there are at least five

alternative nuclear export receptors functioning in higher eukaryotes

that involve signals distinct from the prototypic

leucine-rich NES, are resistant to LMB (29, 62; reviewed in

reference 36), and are predominantly regulated by protein

phosphorylation (25, 46).

* N-VP2 is not involved in nuclear export.. as was suggested previously
* Maturation step in the nucleus involving surface phosphorylations. 🡪 phosphorylations may be crucial for NE since the most prominent difference between both populations

NB, A9 differences 🡪 difference in overall phosphorylation

* Phosphorylation can regulate nuclear export of viruses ..(paper..?)

Nuclear maturation (🡪 EC similar to FC-P1)

FC-P1 precursor of FCP2..?

Two modes nucleophilic (FC-P1), nucleophobic (FC-P2)

* ~~Two distinct populations of DNA containing particles accumulate during the productive infection of MVM in murine fibroblasts.~~
* ~~FC-P1 and FC-P2 are infectious but differ in N-VP2 conformation.~~
* ~~Capsid surface phosphorylation is a key determinant of the different AEX profile.~~
* ~~Only FC-P2 can actively egress from infected host cells.~~
* FC-P1 represents the direct precursor of FC-P2.
* N-VP2 is not involved in the nuclear export of MVM.
* During endocytosis acidic phosphatases remove the FC-P2 surface phosphorylations and change its AEX profile to that of FC-P1.