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Microreview

The way out: what we know and do not know about herpesvirus nuclear egress

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Summary

Herpesvirus capsids are assembled in the nucleus of infected cells whereas final maturation occurs in the cytosol. To access the final maturation compartment, intranuclear capsids have to cross the nuclear envelope which represents a formidable barrier. They do so by budding at the inner nuclear membrane, thereby forming a primary enveloped particle residing in the perinuclear cleft. Formation of primary envelopes is driven by a heterodimeric complex of two conserved herpesviral proteins, designated in the herpes simplex virus nomenclature as pUL34, a tail-anchored transmembrane protein located in the nuclear envelope, and pUL31. This nuclear egress complex recruits viral and cellular kinases to soften the nuclear lamina and allowing access of capsids to the inner nuclear membrane. How capsids are recruited to the budding site and into the primary virus particle is still not completely understood, nor is the composition of the primary enveloped virion in the perinuclear cleft. Fusion of the primary envelope with the outer nuclear membrane then results in translocation of the capsid to the cytosol. This fusion event is clearly different from fusion during infectious entry of free virions into target cells in that it does not require the conserved essential core herpesvirus fusion machinery. Nuclear egress can thus be viewed as a vesicle (primary envelope)-mediated transport of cargo (capsids) through the nuclear envelope, a process which had been unique in cell biology. Only recently has a similar process been identified in *Drosophila* for nuclear egress of large ribonucleoprotein complexes. Thus, herpesviruses appear to subvert a hitherto cryptic cellular pathway for translocation of capsids from the nucleus to the cytosol.

Introduction

Herpesviruses are complex viruses whose mature particles contain in excess of 30 different viral as well as cellular proteins. Herpesvirus particles can be differentiated into four structural components. The linear, double-stranded viral DNA genome is enclosed by an icosahedral capsid composed of at least four capsid proteins. The nucleocapsid is embedded in a proteinaceous layer designated as tegument which is surrounded by a lipid envelope derived from cellular membranes during virion maturation. The envelope carries virally encoded, mostly glycosylated proteins which are required for infection and play important roles as targets for the host's immune response. The tegument is equivalent to the matrix of RNA viruses in linking envelope and nucleocapsid. It is divided into a capsid-associated part ('inner tegument') and an envelope-proximal portion ('outer tegument'). In particular, the composition of the tegument varies between different herpesviruses and is not completely known as it may contain numerous viral and cellular components (Mettenleiter *et al.*, 2009).

The complexity in particle composition is reflected in the complexity of the viral replication cycle. Attachment to cellular surface proteins acting as virus receptors and fusion of the virion envelope with the cellular plasma membrane or, after endocytosis, with the endosomal membrane are mediated by virally encoded envelope glycoproteins. The core fusion machinery, which is conserved throughout the *Herpesviridae*, contains glycoprotein B (gB) and a heterodimeric complex consisting of glycoproteins H and L (gH/gL). By its homology to the fusogenic G protein of vesicular stomatitis virus (VSV), gB is considered the bona-fide herpesvirus fusion protein,

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whereas gH/gL are required for triggering fusion by an as yet unknown mechanism (reviewed in Connolly *et al*., 2011). After fusion incoming nucleocapsids are transported to nuclear pores by the cellular microtubule-associated motor system (Radtke *et al*., 2010) to release the viral DNA through the nuclear pore into the nucleus. Transcription of viral genes as well as viral genomic DNA replication takes place in the nucleus. The formation of progeny viral capsids also occurs in the nucleus after nuclear translocation of capsid proteins which had been synthesized in the cytosol. Capsid formation is similar to head formation of dsDNA-containing bacteriophages by assembly of capsid proteins around a scaffold which is degraded during genome packaging (reviewed in McGeoch *et al*., 2006). Besides mature nucleocapsids (C-capsids), immature capsid forms are observed in herpesvirus infected cells which either are precursors of nucleocapsids still containing the scaffold (B-capsids), or are empty lacking both scaffold and viral DNA after abortive packaging events (A-capsids) (reviewed in Cardone *et al*., 2012).

Escape from the nucleus

While the formation of nucleocapsids is completed in the nucleus, continuing maturation by addition of viral and cellular tegument proteins as well as final envelopment occurs in the cytosol. Thus, herpesvirus nucleocapsids have to cross the nuclear envelope (NE). Compared to the plasma membrane, the NE presents a far stronger physical barrier. The plasma membrane consists of a single membrane which is easily accessed from either side. In contrast, the NE is a membrane system composed of two membranes designated as inner and outer nuclear membranes (INM, ONM) which are separated by the lumen of the perinuclear cleft. The ONM is continuous with the endoplasmic reticulum (ER) and the perinuclear space is continuous with the ER lumen. The NE encloses the nucleus and is only interrupted at sites of nuclear pores. Although there are several theoretical possibilities to traverse the NE for import or export, nucleocytoplasmic transport is usually accomplished exclusively via nuclear pores (reviewed in Antonin *et al*., 2012; Zuleger *et al*., 2012). However, intranuclear herpesvirus capsids display a diameter of *c.* 125 nm. This is too large to cross through intact nuclear pores which include a central basket restricting transport of particles to a maximum size of approximately 36 nm (Panté and Kann, 2002). Moreover, integral NE proteins interact with nuclear lamins and chromatin to form a meshwork obstructing direct access from the nucleoplasm to the INM (reviewed in Hetzer, 2010). Thus, the NE represents a formidable obstacle. Herpesvirus nucleocapsids are largely trapped within this compartment (Movie S1), and only rarely can events be visualized by live-cell imaging that could represent exit of nucleocapsids from the NE (Movie S2).

It has been debated for some time how maturing herpesvirus nucleocapsids actually reach the cytosol (Campadelli-Fiume and Roizman, 2006; Minson and Mettenleiter, 2006). Initially, a single envelopment process had been proposed that included acquisition of an envelope by budding of intranuclear capsids at the INM followed by lumenal transport of these enveloped particles through the ER and the secretory pathway to the cell surface for release (Fig. 1A). This entailed complete assembly of the final viral particle in the nuclear compartment. However, this proposal has been challenged by the finding that major components of enveloped herpesvirus particles in the perinuclear cleft are actually absent from mature virions indicating discontinuity between nuclear and extracellular virions. Alternatively, it had been proposed that herpesvirus nucleocapsids leave the nucleus via widely dilated nuclear pores, thus alleviating the space constraint (Fig. 1B). However, the apparent integrity of nuclear pore assemblies until very late in herpesvirus infection argues against this possibility (reviewed in Mettenleiter *et al*., 2009). Thus, in the last decade a model has been largely accepted on the basis of genetic, biochemical and ultrastructural observations which entails a process designated as nuclear egress. This regulated nuclear egress includes a sequence of envelopment–deenvelopment–reenvelopment (dual envelopment; Skepper *et al*., 2001). In this pathway, intranuclear capsids acquire a (primary) envelope by budding at the INM resulting in the formation of primary enveloped virions residing in the perinuclear space. The primary envelope then fuses with the ONM releasing the capsid into the cytosol for further maturation (Figs 1C and 2, Movie S3). In more general terms, this process could be viewed as a vesicle (= primary envelope)-mediated transport of cargo (= viral capsid) through the nuclear membranes, which has not been described in other circumstances. Thus, nuclear egress has gained much attention. Recent advances in our understanding of the mechanisms behind this process and the gaps in knowledge that still remain will be reviewed here.

The nuclear egress complex (NEC)

In all herpesviruses analysed in this respect, a heterodimeric complex of two viral proteins is required for primary envelope-mediated nuclear egress. Both partners of the complex are conserved throughout the *Herpesviridae*. One component of the heterodimeric complex (Sam *et al*., 2009) is a type II membrane protein tail-anchored in the NE which has been designated as pUL34 in herpes simplex virus 1 (HSV-1) and pseudorabies virus (PrV),

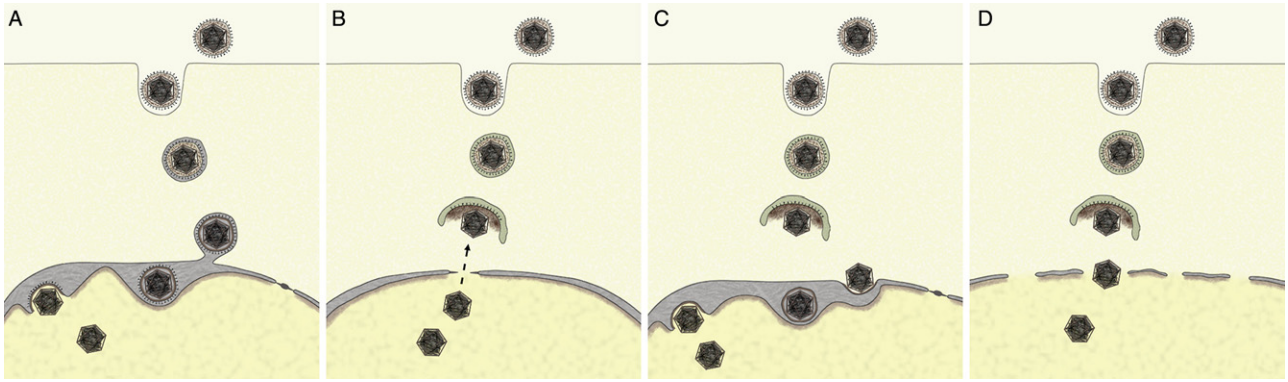

Fig. 1. Diagram of the four models for exit of herpesvirus capsids from the nucleus.
A. Single envelopment model entailing capsid budding at INM and transport via lumenal pathway.
B. Capsid translocation through dilated nuclear pores.
C. Dual envelopment model entailing vesicle-mediated nuclear egress of capsids.
D. Nuclear escape of capsids after nuclear envelope breakdown.
Models B–D require a second envelopment step in the cytosol with acquisition of final envelope including viral envelope proteins (indicated by spikes) and tegument. In model A, all components of the mature virion which is released from the cell have to be incorporated during envelopment at the INM including viral envelope proteins. Virion release from the infected cell occurs by fusion of vesicle and plasma membranes. The different models and their validity are described in the text.

UL50 in human cytomegalovirus (HCMV), M50 in murine cytomegalovirus (MCMV), BFRF1 in Epstein–Barr virus (EBV) and ORF 67 in human herpesvirus 8 (HHV-8). It interacts with a soluble component named pUL31 in HSV-1 and PrV, UL53 in HCMV, M53 in MCMV, BFLF2 in EBV and ORF69 in HHV-8 (reviewed in Johnson and Baines, 2011). For clarity, the HSV-1 and PrV designations will be used henceforth. In the absence of the NEC, nuclear egress is blocked and immature and mature capsids accumulate in the nucleoplasm (reviewed in Mettenleiter *et al*., 2009; Johnson and Baines, 2011). Interestingly, transient or transgenic expression of only the two components of the NEC of PrV (Klupp *et al*., 2007) or HHV-8 (Desai *et al*., 2012) is sufficient to drive formation of correctly sized primary envelopes, i.e. for budding and fission of the primary envelope resulting in accumulation of primary envelopes in the perinuclear cleft which contain the NEC (Hagen *et al*., 2012). It is as yet unclear whether these primary envelopes lacking capsids are also capable of fusing with the ONM, although ultrastructural observations provided indications (Klupp *et al*., 2007).

The NEC also recruits viral and cellular kinases to phosphorylate and, thereby, soften or locally dissolve the nuclear lamina (Muranyi *et al*., 2002; Marschall *et al*., 2011). These include the viral kinases pUS3, which is present only in alphaherpesviruses, and pUL13, which specifies homologues in all herpesviruses (McGeoch *et al*., 2006) as well as cellular kinases, in particular different isoforms of protein kinase C (PKC). pUS3 and pUL13 phosphorylate lamins A and C, whereas PKC preferentially targets lamin B (reviewed in Leach and Roller, 2010; Marschall *et al*., 2011). In addition, other cellular proteins may be involved in nuclear egress such as emerin, an integral INM protein (Morris *et al*., 2007). Thus, besides the core proteins pUL34 and pUL31, the NEC may contain more viral and cellular proteins, but pUL34 and pUL31 are the only viral proteins required for formation of primary envelopes.

The roles of the two partners of the NEC are not yet clear. It is debated whether pUL34 is functionally active beyond providing a membrane anchor for pUL31. Amino acid homologies between the pUL34 homologues are significant in the amino terminal part which has been shown to be relevant for interaction with pUL31, but decrease sharply in the carboxy terminal part. Whereas several studies demonstrated requirement for most of the amino terminal half of pUL34 for interaction with pUL31 (reviewed in Mettenleiter *et al*., 2009; Milbradt *et al*., 2012), others identified a specific internal domain in the amino terminal region of HSV-1 pUL34 necessary and sufficient for pUL31 interaction (Liang and Baines, 2005) or specific residues in the amino terminus of the HCMV homologue (Milbradt *et al*., 2012), which, however, do not overlap. Thus, the molecular basis for the interaction and the interacting interfaces on either protein remain unclear. The non-conserved transmembrane and C-terminal parts of pUL34 can be replaced by transmembrane domains of other pUL34 homologues, of other INM proteins or of unrelated viral or cellular tail-anchored proteins (Ott *et al*., 2011; Schuster *et al*., 2012) without impairing NE localization, pUL31 interaction and function of the NEC. Thus, pUL34 may function as a stalk for proper positioning of pUL31 at the INM. This does not exclude active involvement of pUL34 in the nuclear egress event.

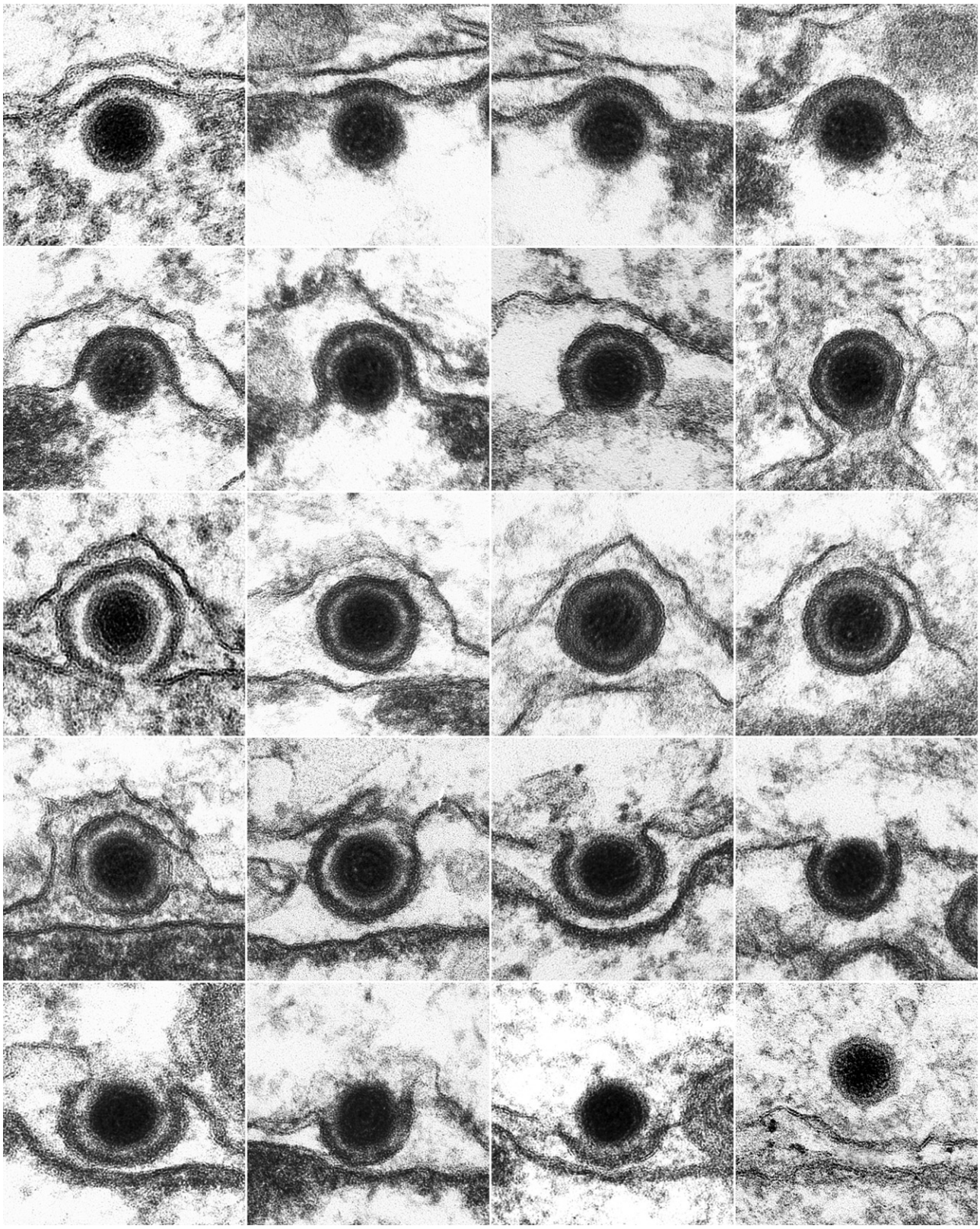

Fig. 2. Series of electron micrographs in support of the envelopment–deenvelopment pathway (animation available as online supporting information). Representative stills from analysis of thin sections of PrV-infected RK13 cells were arranged so that the proposed pathway can be visualized.

Role of the pUL17/pUL25 complex

Softening of the nuclear lamina provides access to the INM, and to the NEC, for the cargo to be transported through the NE, i.e. herpesviral capsids. Intranuclear movement of viral capsids has been associated with nuclear actin filaments which are induced by herpesvirus infection, and nuclear myosin V (reviewed in Taylor *et al*., 2011). After reaching the INM capsids have to dock at the NEC. In several herpesviruses, prominently in PrV (Klupp *et al*., 2011), mature DNA-containing C-capsids are specifically selected for nuclear egress over immature capsid forms. This is, however, not as evident in other herpesviruses. Selection of capsids for envelopment has been proposed to include the complex of the conserved pUL17 and pUL25 proteins (Yang and Baines, 2011), which are two essential minor capsid-associated proteins (Cockrell *et al*., 2011; Toropova *et al*., 2011). The complex had initially been designated as C capsid specific component (CCSC) after nearly exclusive detection on C-capsids (Klupp *et al*., 2006; Trus *et al*., 2007). Thus, interaction between the conserved pUL31/pUL34 complex with the similarly conserved pUL17/pUL25 CCSC could explain preferential recruitment of nucleocapsids into primary envelopes. Functional similarity and at least partial functional overlap have been observed by reciprocal exchange of CCSC components between the alphaherpesviruses PrV and HSV-1 (Kuhn *et al*., 2008; 2010). Subsequently, the pUL17/pUL25 complex has also been detected on immature capsid forms and proposed to be renamed the capsid vertex specific component (CVSC), due to its location at capsid vertices (Toropova *et al*., 2011), which could explain nuclear egress also of immature capsid forms as observed in several herpesviruses. Other interactions between the NEC and the capsid have been described either via pUL33, a conserved protein which is part of the encapsidation machinery (Fossum *et al*., 2009; Vizoso Pinto *et al*., 2011), or by direct contact with the core capsid (Ye *et al*., 2000; Leelawong *et al*., 2011). This, however, does not exclude an interaction between the conserved NEC and CVSC to mediate recruitment of capsids to the primary virion but may be indicative of multiple interactions between the NEC and the capsid.

Composition of the primary enveloped virion

The exact composition of the primary enveloped herpes virion is still unknown. Initially, besides the nucleocapsid presence of the NEC has been demonstrated by immunoelectron microscopy. pUS3 has also been unequivocally detected as part of the primary virion in HSV-1 and PrV. HSV-1 primary virions have been described to also contain viral glycoproteins gB, gD, gH/gL and gM (reviewed in Johnson and Baines, 2011), which were not detected in primary PrV virions (Klupp *et al*., 2008). The same is true for tegument proteins pUL49, pUL41, pUL48 and pUL11. Whether a portion of the inner tegument protein pUL36, which is thought to associate directly with capsids, is actually present in primary virions is debated with evidence for Leelawong *et al*. (2012) and against Möhl *et al*. (2009). However, neither of these proteins is required for formation of primary virions or release of capsids into the cytosol. Moreover, the surface spikes characteristic for mature herpesvirus particles are lacking in perinuclear virions, and the tegument is also morphologically different with a distinct, electron-dense ring closely apposed to the primary envelope (Mettenleiter, 2002). Thus, primary and mature virions differ extensively. Purification of primary virions for an in-depth proteome analysis is hampered by the difficulty to isolate these particles from the perinuclear cleft in high purity, although a respective protocol has been described (Padula *et al*., 2009). However, apparently this is not robust enough to be sufficiently reproducible. A recently developed *in vitro* nuclear egress assay may help to overcome this problem (Rémillard-Labrosse and Lippé, 2011).

Deenvelopment

For translocation of capsids to the cytosol the primary envelope has to fuse with the ONM. Although the existence of this fusion event has now been largely accepted, its molecular details are not understood. In herpesvirus infection, fusion events occur during penetration, nuclear egress and virus release at the plasma membrane. The conserved herpesviral glycoproteins gB and gH/gL are essential for penetration (Connolly *et al*., 2011). Deletion of either one precludes viral infection but does not block nuclear egress or virus release, demonstrating that these fusion events are mechanistically different. In fact, nuclear egress of HSV-1 and PrV occurs in the absence of the core entry fusion machinery encompassing gB and gH/gL, although it was shown to be impaired in the simultaneous absence of gB and gH in HSV-1 (Farnsworth *et al*., 2007) but not in PrV (Klupp *et al*., 2008). Thus, the entry fusion machinery may modulate nuclear egress but is not required for this process. Therefore, other components of the primary virion may be involved. pUL34 is the only transmembrane protein unequivocally identified as constituent of primary virions in different herpesviruses. Thus, it could participate in fusion during nuclear egress. However, the very short predicted lumenal domains of pUL34 homologues, encompassing, e.g. only one amino acid in PrV, reside in the non-conserved part of the protein and can be substituted by heterologous sequences (Ott *et al*., 2011; Schuster *et al*.,

2012). This argues against a direct involvement of the NEC in deenvelopment. Thus, it has been postulated that other, presumably cellular, proteins could mediate fusion of the primary envelope and the ONM, and hence the hypothesis has been forwarded that herpesviruses actually subvert a cryptic cellular fusion machinery associated with the NE (Klupp *et al*., 2008). However, it is undisputed that the pUS3 kinase is involved in nuclear egress of alphaherpesviruses, since in its absence primary enveloped virions accumulate in the perinuclear cleft. HSV-1 pUS3 has been demonstrated to phosphorylate pUL31 (Mou *et al*., 2009) and the cytoplasmic domain of gB (Wisner *et al*., 2009) which may in part explain the observed modulation of nuclear egress, although the exact mechanism remains unclear. Overexpression of cellular torsin A, an AAA+ ATPase, also impairs deenvelopment leading to accumulation of primary virions (Maric *et al*., 2011).

Herpesvirus-induced NE breakdown

Primary envelope-mediated nuclear egress is a highly regulated process which does not impair integrity of the NE even late in herpesvirus infection. It requires the NEC and is blocked in the absence of either NEC partner. However, in PrV infectivity is not eliminated in the absence of the NEC, although viral titres decrease by *c.* 10^3- to 10^4-fold. This residual infectivity was used for reversion analysis of PrV mutants lacking either pUL34 or pUL31. After extensive passaging on rabbit kidney (RK13) cells, mutant viruses were recovered from either assay which replicated with wild-type kinetics and titres. Ultrastructural analyses demonstrated that infection by these viral mutants resulted in breakdown of the NE leading to leakage of nucleoplasmic capsids into the cytosol (Fig. 1D; Klupp *et al*., 2011). Cytoplasmic morphogenesis of these capsids proceeded resulting in the formation of infectious virions. However, the selectivity for C-capsids as observed during nuclear egress is lost after NE breakdown. Comparative sequence analysis of the two mutant viruses disclosed several congruent mutations which included envelope and tegument proteins as well as the viral maturational protease. Mutations in tegument proteins involved in signalling via the exogenous signal-regulated kinase ERK and inhibitor studies targeting ERK signalling indicate involvement of this cellular signalling pathway in herpesvirus-induced NE breakdown (Grimm *et al*., 2012). It is tempting to speculate that herpesviruses have actually developed a means to maintain integrity of the nucleus until late in infection to prevent premature breakdown of cellular compartmentalization or activation of innate, cytoplasmic DNA-sensing immune responses (Rasmussen *et al*., 2011) with detrimental effects on viral replication. Thus, there may be alternatives to vesicle-mediated nuclear egress. Interestingly, other viruses which are faced with the same problem of overcoming the NE also effect disruption of the NE. The simian virus 40 late viral protein VP4 is a viroporin (Raghava *et al*., 2011) which disrupts the NE for viral release (Giorda *et al*., 2012). In the opposite direction, incoming parvovirus particles enter the nucleus through small, transient disruptions of the NE (Cohen *et al*., 2011).

Role of the NEC beyond nuclear egress

The role of the NEC in nuclear egress is well established. However, either the NEC or its components may play roles in herpesvirus replication beyond nuclear egress. Thus, mutations have been identified in pUL34 which affect viral cell-to-cell spread and trafficking of glycoprotein E (Haugo *et al*., 2011). Conversely, mutations have been described which do not support efficient nuclear egress but partially restore plaque formation (Schuster *et al*., 2012). Intra- and extragenic suppressor mutations of a charge cluster mutant of HSV-1 pUL34 also point to multiple functions of pUL34 (Roller *et al*., 2011). These observations indicate that at least pUL34, but perhaps the whole NEC, may be involved in other processes during herpesvirus infection.

Nuclear egress of cellular ribonucleoprotein (RNP) complexes

Vesicle-mediated nucleocytoplasmic transport has been unknown in cell biology except in herpesvirus infection. Thus, it had been questioned for a long time whether this process actually occurs or results from misinterpretation of observations (Campadelli-Fiume and Roizman, 2006; Mettenleiter and Minson, 2006; Minson and Mettenleiter, 2006). Only recently, exciting results indicate that vesicular transport from the nucleus to the cytosol may be more widespread than hitherto known. In *Drosophila* synapse differentiation at neuromuscular junctions, nuclear import of a proteolytic fragment of the plasma membrane receptor *Drosophila* frizzled-2 (DFz2) results in colocalization of the fragment DFz2C in characteristic foci at the nuclear periphery with lamin C and large RNP complexes. These RNP complexes contain mRNA coding for postsynaptic proteins. Formation of the DFz2C complexes requires lamin C and PKC, and compelling evidence indicates that these mRNP complexes leave the nucleus by a nuclear membrane budding fusion mechanism similar to herpesvirus nuclear egress (Speese *et al*., 2012). This supports the assumption that membrane budding is a general cellular mechanism for export of mRNPs and other large cargo from the nucleus, and that herpesviruses take advantage of this preexisting, hitherto cryptic host pathway for nuclear egress (Hatch and Hetzer, 2012;

Montpetit and Weis, 2012). It remains to be analysed how these mRNP complexes are recruited to the vesicle, which cellular proteins mediate vesicle formation and fusion and which requirements nuclear cargo has to fulfil to access this intracellular vesicle-mediated transport pathway. During herpesvirus nuclear egress primary envelopment, i.e. recruitment of cargo (= capsids) to the vesicle, and vesicle (= primary envelope) formation are dependent on the NEC, whereas deenvelopment may actually be dominated by the cellular machinery.

Conclusion

Herpesviruses use a vesicle-mediated transport for translocating intranuclear capsids to the cytosol for continuing virion maturation. This process is mechanistically incompletely understood but both viral and cellular proteins play important roles in its execution. Considered herpesvirus-specific for a long time, recent observations open the exciting prospect that vesicle-mediated transfer of cargo through the NE is actually a hitherto unknown way of nucleocytoplasmic transport in normal cellular metabolism. This reemphasizes the validity of using viruses to uncover cellular functions, and supports the hypothesis that herpesviruses subvert a preexisting cellular pathway for securing nuclear egress of nascent capsids and, thus, their replication. It remains to be seen whether other viruses which are faced with the requirement to traverse the NE as, e.g. baculo- (Yuan *et al*., 2011) or nucleorhabdoviruses (Bandyopadhyay *et al*., 2010) also use this transport pathway.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Fluorescently labelled nucleocapsids (red) within the nucleus. PrV capsids were labelled by fusion of autofluorescent mCherry protein with the non-essential pUL35 decorating the hexons of the capsid. The nuclear envelope was labelled by expression of a fusion protein of GFP and LAP2β which was constitutively and stably expressed in the constructed transgenic rabbit kidney (RK13) cell line. Confocal time lapse images of one z-slice were taken with a Leica TCS SP5 microscope. Time intervals are indicated. Scale bar = 2 μm.

Movie S2. Fluorescently mCherry-labelled nucleocapsid (red) presumably exiting from the nuclear envelope of a RK13 cell labelled by stable, transgenic expression of EGFP–lamin B1. Left: Live-cell images taken with a Leica TCS SP5 microscope. Maximum intensity projection of five z-slices (503.54 nm). Right: 3D reconstruction of lower left part at different angle to improve visibility of the exiting particle. See particle exiting at lower left.

Movie S3. Electron micrographs from Fig. 2 have been arranged and animated so that the proposed pathway can be visualized. Artificial animation has been chosen to give a visual impression of the proposed egress pathway but does not represent 'time lapse' live video photography.