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Nuclear Exodus: Herpesviruses Lead the Way

Janna M. Bigalke and **Ekaterina E. Heldwein**

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Ekaterina E. Heldwein: katya.heldwein@tufts.edu

Abstract

Most DNA viruses replicate in the nucleus and exit it either by passing through the nuclear pores or by rupturing the nuclear envelope. Unusually, herpesviruses have evolved a complex mechanism of nuclear escape whereby nascent capsids bud at the inner nuclear membrane to form perinuclear virions that subsequently fuse with the outer nuclear membrane, releasing capsids into the cytosol. Although this general scheme is accepted in the field, the players and their roles are still debated. Recent studies illuminated critical mechanistic features of this enigmatic process and uncovered surprising parallels with a novel cellular nuclear export process. This review summarizes our current understanding of nuclear egress in herpesviruses, examines the experimental evidence and models, and outlines outstanding questions with the goal of stimulating new research in this area.

Keywords

viral egress; nuclear egress; envelopment; de-envelopment; budding; scission; herpesvirus; structure

INTRODUCTION

Viral egress is the process of viral escape from the cell that is frequently coupled with viral biogenesis. For enveloped viruses, this process must involve the acquisition of a lipid envelope. This is typically accomplished by capsid budding at a cellular membrane. Different viruses have evolved specific strategies for budding, not only with distinct mechanisms but also locations. In some viruses (e.g., retroviruses, paramyxoviruses, orthomyxoviruses, arenaviruses, filoviruses, coronaviruses, and some rhabdoviruses) capsids bud at the plasma membrane; in others (e.g., flaviviruses, foamy viruses, and vaccinia virus), they bud at intracellular membranes, such as the endoplasmic reticulum (ER) or Golgi (reviewed in 1). In the former case, budding accomplishes both exit from the cell and morphogenesis in one fell swoop: escaping confines of the cell and acquiring an envelope. But in the latter case, budded virions, being intracellular, must yet undergo maturation in the cytoplasm and find a way out of the cell. Hijacking the secretory pathway is the typical way

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for the enveloped particles to get out, and a number of viruses use this pathway to complete their morphogenesis by modifying surface proteins in secretory compartments so as to prime the virion for the subsequent infection of a new cell.

In the above cases, viruses (with the exception of orthomyxoviruses) replicate entirely in the cytoplasm. Most DNA viruses, however, replicate in the nucleus, e.g., baculoviruses, polyomaviruses, and adenoviruses (reviewed in 2). They have to escape from the nucleus first, before they undergo maturation in the cytoplasm. They need to cross the nuclear envelope, which consists of two lipid bilayers, and the perinuclear space, which is contiguous with the ER. Influenza virus has a significant nuclear replication stage, but after replication, genome segments are thought to exit the nuclei through the nuclear pores and then follow the example of cytoplasmic viruses. Nonenveloped DNA viruses, such as adenoviruses and papovaviruses, do not need to acquire an envelope and are thought to escape the nucleus by inducing cell lysis, during which the nuclear envelope is disrupted. Herpesviruses present an interesting case. These viruses replicate and encapsidate their genomes in the nucleus, but their capsids are too large to pass through the nuclear pores. Viral replication is not typically accompanied by cell lysis. How do the capsids get out of the nucleus and the cell and in the process acquire their envelopes? The answer may seem surprising: Herpesvirus capsids bud twice, first at the nuclear envelope and then into cytoplasmic membranes. Two rounds of budding do not result in a double envelope; instead, the first envelope is lost at the last stage of nuclear egress where it fuses with the outer nuclear membrane. Only the second, and final, round of budding in the cytosol generates the single-layer envelope of the mature virus.

The goal of this review is to present the current knowledge of herpesvirus nuclear egress, with a focus on the recent groundbreaking discoveries, and the many questions, some lingering and others emerging from the latest findings. We hope to entice the readers to examine and, perhaps, reconsider some of the currently accepted models and to stimulate debate. Most importantly, this review aims to inspire further research to shed light on the many mysteries of herpesvirus egress in particular and nuclear egress in general.

OVERVIEW OF HERPESVIRUS EGRESS

Herpesviruses are double-stranded DNA, enveloped viruses that infect nearly all vertebrates and even bivalves. These viruses are divided into three subfamilies, alpha-, beta-, and gammaherpesviruses. Eight human herpesviruses establish lifelong latent infections and cause a plethora of ailments from skin lesions and ocular diseases to encephalitis, cancers, congenital infections, and disseminated disease in the immunocompromised people, e.g., organ transplant recipients or AIDS patients.

Herpesvirus genomes are replicated and encapsidated in the nucleus (3). However, final stages of maturation occur in the cytoplasm, which means that capsids have to get out of the nucleus into the cytoplasm. The nucleus is surrounded by a double membrane of the nuclear envelope, and most traffic in and out of the nucleus occurs through the nuclear pores, which have a diameter of ~39 nm (4), but at a ~125-nm for herpes simplex virus (HSV)-1, capsids cannot fit through the pores. Instead, they have evolved a laborious mechanism to cross the

nuclear envelope (reviewed in 5, 6), whereby capsids first bud at the inner nuclear membrane in a step called primary envelopment (Figure 1). Once in the perinuclear space, these primary viral particles fuse their membranes with the outer nuclear membrane, releasing capsids into the cytoplasm, a step called de-envelopment. These cytoplasmic capsids next travel along microtubules to vesicles derived from the *trans* Golgi network or endosomes (5), where a second, and final, round of envelopment takes place. There, capsids acquire their lipid envelope and the tegument—a protein layer between the capsid and the envelope. Cellular secretory machinery then transports these mature virions out of the cell.

The current envelopment/de-envelopment/re-envelopment model of the herpesviral egress has become generally accepted only within the last 10–15 years. Initially, an egress process with a single round of budding was favored (7; reviewed in 8). It was thought that capsids underwent a single round of budding at the inner nuclear membrane, and given that the perinuclear space is contiguous with the ER lumen, it made sense for the resulting virions to be then transported from the ER lumen to the extracellular space via the secretory pathway. In this manner, the herpesviral capsids were thought to bypass the cytosol. Admittedly, this single-budding model is more intuitive then the far less obvious double-budding model. However, multiple experimental findings provide strong support for the latter model. For example, deletion of a HSV-1 or pseudorabies virus (PRV) US3 kinase (9–11) causes perinuclear virions to accumulate in the perinuclear space, causing formation of extensive herniations of the perinuclear space that expand to accommodate stuck perinuclear virions. Furthermore, deletions of certain tegument proteins and glycoproteins result in accumulation of naked capsids in the cytoplasm (12–18). Some of the mature virion components are not present in either the perinuclear viral particles or in the nucleus itself (19–22), and some of the major components of the perinuclear virions, such as UL31 and UL34, which are discussed below, are not incorporated into mature extracellular virions (9, 23). In addition, the morphology of the perinuclear virions and mature virions is markedly different; the former has a thin tegument layer and relatively smooth envelope, and the latter has a thick tegument and clear glycoprotein spikes (20, 21), which implies that the two particles are products of two independent budding events. Finally, the envelope of the mature extracellular virions is derived from cytoplasmic rather than nuclear membranes (21, 22). All of this evidence strongly argues in favor of a mechanism whereby nuclear capsids acquire their envelope at the inner nuclear membrane, naked capsids escape into the cytoplasm, and the final envelope is acquired at cytoplasmic vesicles. Although this envelopment/de-envelopment may not be the only possible route out of the nucleus, it appears to be the dominant mechanism in herpesviruses.

PRIMARY ENVELOPMENT (NUCLEAR BUDDING)

Capsid budding at the inner nuclear membrane is a complex process that involves many viral and cellular participants, some yet to be identified. Recent advances in the field have led to a general model of this process. For the capsid to bud into the inner nuclear membrane, the following activities have to take place:

- The nuclear lamina has to be dissolved.
- The capsid needs to be recruited to the inner nuclear membrane.

- The capsid has to wrap into the membrane.
- The nascent bud must be cleaved so that the vesicle can be released on the opposite side of the membrane, into the perinuclear space.

Nuclear Envelope

The nuclear envelope consists of the inner nuclear membrane; the perinuclear space, which is continuous with the ER; and the outer nuclear membrane. The distance between inner and outer nuclear membranes is 50 nm, which is maintained by the linker of nucleoskeleton and cytoskeleton (LINC) complex containing Sad1p, UNC-84 (SUN) and Klarsicht, ANC-1, Syne homology (KASH) domain proteins (reviewed in 24). LINC is a multiprotein complex that directly connects the nucleoskeleton to the cytoskeleton. The nuclear envelope is further stabilized by a dense fibrillar network inside the nucleus, which consists of lamins and other membrane-associated proteins (reviewed in 25). The three types of lamins (A, B, and C) assemble into intermediate filaments and provide mechanical support. Lamin B associates with the inner nuclear membrane and provides essential support for the nucleus (26). It interacts with integral membrane proteins, such as the lamin B receptor and emerin, thereby tethering the nuclear lamina to the inner nuclear membrane (27). Lamins A and C, which are the products of alternative splicing of the *LMNA* gene, add stiffness to the nuclear envelope (reviewed in 25).

The Nuclear Egress Complex

Two conserved viral proteins are the major players in nuclear egress of all herpesviruses. In alphaherpesviruses, these proteins are called UL31 and UL34, and together they compose the nuclear egress complex (NEC). There are orthologs of UL31 and UL34 in all herpesviruses, e.g., UL53 and UL50 in human cytomegalovirus (HCMV, a betaherpesvirus), BFLF2 and BFRF1 in Epstein-Barr virus (EBV, a gammaherpesvirus) and ORF69 and ORF67 in Kaposi's sarcoma–associated virus (KSHV, a gammaherpesvirus). In this review, we use the alphaherpesvirus nomenclature to describe the sequence of events but point out differences for other herpesviruses. Both UL31 and UL34 are essential for nuclear egress. In the absence of either protein, capsids are retained in the nucleus and do not bud at the inner nuclear membrane. As the result, viral titers drop dramatically (23, 28–33). UL31 is a soluble phosphoprotein (32, 33) that localizes to the nucleoplasm in the absence of UL34. UL34 has a C terminal transmembrane helix that anchors it in the inner nuclear membrane with only three residues (in HSV-1) extending into the perinuclear space (34). Formation of the NEC is a prerequisite for proper localization of both UL31 and UL34 in the inner nuclear membrane as well as for nuclear egress (29, 35–37).

Crystal Structure of the Nuclear Egress Complex

The crystal structures of the NEC from HSV-1, PRV, and HCMV revealed an elongated complex of approximately 80 × 40 × 40 Å (Figure 2) (38–41). UL34 has a globular fold and forms a pedestal. UL31 also has a globular core that sits on top of the UL34 pedestal, but it features an N-terminal hook-like extension that reaches the opposite end of the NEC while wrapping around one margin of UL34. All crystallized NEC versions are missing the unstructured N terminus of UL31 (17–50 residues, depending on the virus) and the C

terminus of UL34 (85–222 residues, depending on the virus), which includes residues necessary for membrane interactions in UL31 and UL34 and the transmembrane anchor of UL34. Based on the location of the last resolved residues abutting the membrane-interacting regions in UL31 and UL34, the UL34 pedestal is at the membrane-proximal end of the NEC structure, whereas the helical cap in UL31 is at the membrane-distal end.

UL31 and UL34 interact extensively via two distinct interfaces. The larger interface is mediated by the N-terminal V-shaped α-helical hook of UL31, which wraps around the globular UL34, and the smaller interface is created by contacts between the globular portions of the two proteins. Extensive interactions render the soluble portion of the NEC into a very stable and possibly conformationally restricted complex, whereas the membrane-binding N terminus of UL31 and C terminus of UL34, which are unresolved in the crystal structures, are likely flexible. High predicted affinity between UL31 and UL34 suggests that at the inner nuclear membrane of infected or transfected cells UL31 and UL34 mostly exist as a complex.

We now focus on the steps leading up to the budding process itself, including lamina dissolution, capsid recruitment, and membrane deformation, all of which require the NEC (Figure 3).

Lamina Dissolution

In order for the viral capsid to access the inner nuclear membrane, the barrier presented by the nuclear lamina meshwork has to be dissolved. During mitosis, the lamina disassembles between prophase and metaphase and reassembles during interphase (42). Complete lamina disassembly is achieved by site-specific, reversible phosphorylation of lamins by the cellular kinases protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and cell division cycle 2 (Cdc2) (43–45). But during herpesvirus infection, there is no massive nuclear lamina disassembly, and the lamina is only disrupted in areas with high concentrations of the NEC (46–49). Not surprisingly, UL34 and its homolog in betaherpesviruses can recruit PKCα and PKCδ, both of which can phosphorylate lamin B during a herpesvirus infection (30, 48, 50). These cellular kinases are needed for efficient nuclear egress; however, their recruitment is insufficient, and additional viral kinases are required in the lamina dissolution. All herpesviruses encode homologs of the alphaherpesvirus UL13 kinase (UL97 in HCMV, BGLF4 in EBV, ORF36 in KSHV) (51), whereas alphaherpesviruses also encode a second kinase, US3 (52). US3 phosphorylates lamin A/C (53). Although US3 contributes to efficient nuclear egress in alphaherpesviruses, it appears more important for the de-envelopment step rather than the primary envelopment (10). UL13 does not appear necessary for nuclear egress in HSV-1 (54), but it is important for the conformational alteration of lamina components in HSV-2 (49).

UL13 homologs play central roles in nuclear egress in beta- and gammaherpesviruses (55–58). In HCMV, inhibition or deletion of UL97 results in a defect in lamina disruption and a 100-fold reduction in replication efficiency (59). HCMV UL97 is recruited to sites of primary envelopment by the UL34 homolog UL50 (57). This creates a binding site for PIN1, which induces additional conformational changes in the lamina (60) that may be required for an overall reorganization of lamin proteins. The UL13 homolog in EBV also phosphorylates

lamin A/C (56). How kinase activity is regulated is unclear, but it is possible that only recruitment to the inner nuclear membrane by the NEC activates viral and cellular kinases. UL13 homologs in beta- and gammaherpesviruses have been described as Cdc2-like because they target the same site in lamin A/C, which further indicates a role in lamina dissolution as Cdc2 is very important in lamin phosphorylation during mitosis (see above) (55, 61, 62).

Outside of kinase recruitment for the phosphorylation of lamins, the NEC also binds directly to lamin A/C, thereby competing with the binding of the lamins to each other. This disrupts the fibrillar network of the nuclear lamina (46, 63). Additionally, NEC binding also perturbs interaction of lamins with emerin, which links the lamina to the inner nuclear membrane (64, 65). These interactions further weaken the stiffness of the nuclear lamina. Thus, the NEC in all herpesviral subfamilies is essential for the first step in nuclear egress, the dissolution of the lamina layer, necessary for the capsid's approach to the inner nuclear membrane.

Capsid Recruitment to the Inner Nuclear Membrane

Herpesviral capsids are icosahedral, with $T = 16$ symmetry, and consist of the major capsid protein, VP5 (UL19) in alphaherpesviruses, arranged into 11 pentons and 150 hexons, which are linked by triplexes, composed of UL18 (VP23) and UL38 (VP19C) (reviewed in 66). UL35 (VP26) forms a hexameric ring on each hexon (67). Each capsid also contains a single portal, which occupies 1 of the 12 vertices and is composed of 12 copies of the portal protein UL6 (68, 69), through which viral DNA is encapsidated (68). Assembly begins with the formation of a spherical procapsid; formation of the procapsid requires the presence of multiple copies of the viral protease-scaffold protein UL26, which is subsequently self-digested and replaced by viral DNA in mature capsids (reviewed in 66). In infected cells, three types of capsids can be observed: A, B, and C capsids (reviewed in 66). C capsids are the mature capsids containing viral DNA genomes. B capsids contain the scaffold, and A capsids are empty. Whether A and B capsids represent dead-end products of capsid assembly (70) or assembly intermediates (66) is debated. All capsid types are found in the nucleus, whereas in the cytoplasm, mainly C capsids are found. The capsid vertex-specific complex consists of the auxiliary capsid proteins UL17 and UL25 and ensures proper DNA retention in the mature capsid (71, 72), likely by stabilizing the capsid to help it withstand high internal pressure from encapsidated DNA. Initially, the capsid vertex-specific complex was found only on mature C capsids (73), but it was later also identified on A and B capsids, albeit in lower amounts (74).

Nuclear capsids probably get recruited to the inner nuclear membrane from the replication sites by binding UL31 (35, 75). UL31 can bind a complex of UL25 and UL17 even in the absence of capsids (76) and may interact with capsids either by binding these proteins or the major capsid protein VP5 (75, 77), but this has not yet been conclusively shown. The highly conserved surface patch on the membrane-distal end of UL31, identified in the HSV-1 NEC crystal structure (Figure 2) (38), is the likely location of the capsid-binding site. Infected nuclei contain free UL31 (not a part of the NEC) within the nucleoplasm, and it has been proposed that this free UL31 binds capsids and then hands them over to the NEC at the inner nuclear membrane (35).

C capsids are the dominant species undergoing envelopment at the inner nuclear membrane (78; reviewed in 5), implying that primary envelopment is used as a quality-control mechanism to ensure that only mature, DNA-filled capsids can exit the nucleus. The selection mechanism is currently unclear. Earlier, UL25 and its binding partner on the capsid surface, UL17, were proposed to function as the selectivity determinants because at that time they were only found on C capsids. However, their presence on A and B capsids suggests that they are not the sole determinants of the selection mechanism. Given that the three capsid types have distinct structures (reviewed in 66), it appears likely that C capsids are selected based on preferential binding of UL31 to a specific conformation or a posttranslational modification of certain capsid proteins, such as UL25 or VP5.

Membrane Deformation and Budding by the Nuclear Egress Complex

Once the capsid is recruited to the inner nuclear membrane, budding involves membrane deformation around the capsid, followed by pinching off the nascent bud. The resulting vesicle then ends up in the perinuclear space. Both UL31 and UL34 are essential for this process because, in the absence of either protein, capsids are retained in the nucleus and the viral titers drastically drop (23, 28). In addition to its roles in lamina dissolution and capsid recruitment to the nuclear envelope, the NEC is also thought to play a direct role in nucleocapsid budding at the inner nuclear membrane. Transient coexpression of UL31 and UL34 homologs from PRV, KSHV, or EBV in transfected cells was sufficient to drive formation of perinuclear vesicles (79–81) or multilayered ruffles in the nuclear envelope (36).

Although these experiments showed that no other viral proteins besides UL31 and UL34 were necessary for vesiculation, they did not address the specific function of the NEC in membrane deformation. Did it serve to recruit one or several cellular membrane-manipulating proteins, with no direct role in membrane deformation, or was the complex capable of mediating membrane deformation and budding directly? This question was answered when recombinant HSV-1 NEC was shown to vesiculate synthetic lipid membranes in the absence of any other components or chemical energy (82). The NEC was not only capable of driving bud formation in vitro but also of mediating scission. These findings for the first time clearly demonstrated the intrinsic membrane-budding ability of the NEC. This ability was subsequently confirmed for the PRV NEC (83) and is expected to be conserved among other herpesviruses given the conservation of the NEC sequence and structure and its universal requirement for primary envelopment.

The Nuclear Egress Complex Mediates Budding by Assembling into a Hexagonal Lattice

The NEC forms ordered coats, characterized by hexagonal symmetry, on the inner surface of budded vesicles (Figure 4). These honeycomb-like coats were first observed on the inner surface of budded vesicles obtained in vitro (82) and later within perinuclear vesicles in transfected cells (84). In vitro these coats form rapidly; no energy in the form of ATP is required, and coat formation is driven exclusively by NEC-NEC and NEC-membrane interactions. Cryoelectron tomography of infected nuclei provided snapshots of the budding process (84). The NEC forms ~100-nm hexagonal patches as the nucleocapsid approaches the inner nuclear membrane. These patches expand as the budding process progresses until a

complete hexagonal coat is formed around the capsid. These observations suggest that the NEC drives budding by forming a hexagonal coat that efficiently scaffolds the membrane from the inside.

In cells transiently expressing UL31 and UL34, empty perinuclear vesicles have an average inner diameter of ~100–117 nm (84). Capsids, however, are slightly larger (~125 nm), which suggests that the capsid may determine the size of primary viral particles. This would also point to a very close contact between the NEC and nucleocapsids.

An atomic-level view of the hexagonal lattice was provided by the crystal structure of the HSV-1 NEC, which crystallized in the hexagonal space group P6 (38). The NEC lattice is built from hexameric rings with a diameter of ~110 Å (Figure 4). This arrangement agrees very well with the geometry and dimensions of the HSV-1 NEC coats formed on the inner surface of vesicles budded in vitro (82) and the perinuclear vesicles formed in PRV NEC-expressing cells (84). Interactions at the hexameric interfaces are extensive and conserved, supporting the idea that it is the building block of the honeycomb lattice. The NEC lattice is formed by interactions that involve both UL31 and UL34. Mutations that disrupt hexamers or their packing within the lattice (intra- and interhexameric contacts, respectively) reduce budding in vitro (38) and nuclear egress in infected cells (85, 86); these activities demonstrate that NEC oligomerization into hexagonal arrays is the driving force for NEC-mediated budding. A similar hexagonal lattice was observed in the crystals of the HCMV NEC (40), which suggests that the ability to oligomerize into hexagonal array is a conserved property of the NEC.

Our understanding of the nuclear budding mechanism indicates that the NEC is a stable complex that functions as one unit, and the NEC lattice is formed by interactions that involve both UL31 and UL34. Recently, PRV UL31, N-terminally tagged with a tandem His-EGFP tag, has been reported to vesiculate synthetic Ni-NTA-containing liposomes in the absence of UL34 in vitro (83) but not in vivo (87). This UL34-independent budding activity of tagged UL31 is difficult to reconcile with the observation that certain UL34 mutants, described previously, are nonfunctional despite the ability to form the NEC and to localize to the inner nuclear membrane (85, 86). In the HSV-1 NEC crystal structure, these UL34 mutations map to the oligomerization interfaces (38), and their nonfunctional phenotype can be explained by the disruption of the NEC lattice and, thus, budding. Furthermore, no hexagonal lattice was observed for UL31 alone (83), so the ability of UL31 to mediate membrane budding remains unexplained.

How Does the Nuclear Egress Complex Form Spherical Coats?

The hexagonal crystal lattice formed by the NEC is flat, whereas the honeycomb coats are spherical. Although the geometry and dimensions of the flat crystal lattice and the curved vesicle coats are similar, the coat cannot be too rigid because it must be able to accommodate curvature while maintaining local hexagonal symmetry. Cryo–electron microscopy analysis of the PRV NEC coat on the inner nuclear membrane before and after budding suggests that the membrane-proximal regions of the NEC are very flexible (84), providing a mechanism for generating curved arrays. The flexible C terminus of UL34 may

specify the degree of curvature because when it is truncated the size of the budded vesicles is more variable (82) than when it is intact (79).

But such conformational flexibility is not the only requirement for generating a spherical object. Strictly symmetrical hexagonal packing would result in a flat array, whereas formation of a sphere requires distortions in hexagonal packing. This is commonly achieved through a regular inclusion of pentagons into a hexagonal lattice, which generates icosahedrons (think soccer balls and viral capsids). The NEC coats lack icosahedral symmetry (82, 84). However, curvature could also arise from incorporation of irregular defects into a hexagonal lattice, as can be observed in the immature HIV capsids formed by Gag (88, 89) and the early poxvirus envelope (90) formed by D13 (91). Higher-resolution cryo–electron microscopy images of the NEC coats are required to visualize the geometry of the NEC coat in detail.

Interestingly, both immature HIV capsids and early poxvirus envelopes, each composed of hexagonal coats containing irregular defects, are transient and not retained in mature viral particles. The immature HIV capsid is converted into a mature capsid characterized by broken icosahedral symmetry (88). The early poxvirus envelope formed by D13, thought to drive the formation of membrane crescents, is disassembled shortly after its coalescence into spherical particles (92). Likewise, the NEC coat is disassembled during de-envelopment (22). It is tempting to speculate that hexagonal coats containing irregularities possess intrinsic properties that allow easy disassembly.

How Does the NEC Mediate Scission?

The NEC has an intrinsic ability to mediate both budding and scission (82). Although the NEC scaffolds the bud by forming a hexagonal coat on the membrane, the scission mechanism is less clear. The openings in the lattice are ~5 nm in diameter, and at this thickness, the neck could undergo spontaneous scission. Thus, the formation of the lattice alone could narrow the neck to the point of scission. This scission mechanism differs from that of the cellular endosomal sorting complex required for transport (ESCRT)-III proteins, which localize to the neck of the budding vesicle and may constrict it by forming a spiral-shaped polymer from its exterior end (93, 94). Alternatively, the NEC may use a different, as-yet-uncharacterized, mechanism to mediate scission.

Although the NEC constitutes a minimal membrane budding machinery and could, in principle, carry out budding in infected cells without the need for host factors, this does not exclude the possibility that it recruits a cellular factor to assist in neck scission. If so, the NEC would resemble the HIV matrix protein Gag, which forms a coat on the inner surface of the nascent bud. Although Gag is capable of assembling into virus-like particles in vitro (95), viral budding requires cellular ESCRT-III proteins, which are recruited to mediate scission (96). In the case of the NEC, nuclear ESCRTs, which are important during nuclear envelope reformation during telophase (reviewed in 97), could fulfill this function. Although nuclear egress of HSV-1 is insensitive to the dominant negative mutant of the AAA-type ATPase Vps4 (98), which is a well-accepted test for ESCRT involvement, nuclear ESCRTs have been reported to colocalize with a different AAA-type ATPase, p97, at sites of annular fusion (99).

AAA-type ATPases torsins, which reside in the perinuclear space and the ER lumen, have been implicated in nuclear budding of HSV-1 (100). However, knockout of torsinA and torsinB leads to only a twofold drop in a herpesvirus titer (101), suggesting that, although torsins may increase the efficiency of nuclear budding, they are not essential, which is consistent with the ability of the NEC to mediate budding on its own. Overexpression of torsinA decreases herpesvirus replication by fivefold and leads to aberrant accumulation of perinuclear viral vesicles inside the ER (100). Torsin may regulate the localization of KASH and SUN domain–containing proteins that maintain the stability of the perinuclear space and enable regular spacing of the inner nuclear membrane–outer nuclear membrane distance as part of the LINC complex (102). Excess of torsinA may cause these proteins to mislocalize, allowing perinuclear virions, normally confined to the perinuclear space, to leak into ER.

Nuclear Egress Complex Activity Needs to Be Controlled

The NEC has powerful membrane vesiculation activity in transfected cells (79) as well as in vitro (82). Yet empty perinuclear vesicles are rarely observed during infection (5, 6), and primarily mature C capsids bud into the inner nuclear membrane (78). Therefore, in infected cells, the intrinsic budding potential of the NEC has to be under tight control to prevent unproductive budding. Given that NEC oligomerization is the driving force for vesiculation, formation of the NEC lattice must be inhibited until the arrival of mature capsids and must be triggered by proteins or certain protein conformations present on C capsids but not on A or B capsids to ensure that only DNA-containing capsids exit from the nucleus. Neither the inhibitory nor the triggering mechanism has been elucidated, but available data offer several possibilities. First, the NEC should be present at the inner nuclear membrane in an inactive form, either bound to an unidentified inhibitory viral protein or altered by posttranslational modifications, such as phosphorylation. During infection, proteins present on mature but not immature capsids may trigger oligomerization either by binding the NEC directly or by inactivating an inhibitor that blocks NEC oligomerization. Phosphorylation of the HSV-1 UL31 by the viral kinase US3 may inhibit its budding activity (103), whereas dephosphorylation would release the inhibition and trigger NEC oligomerization. In this scenario, C capsids or proteins associated with them would need to have a phosphatase activity, but no phosphatase has been implicated in nuclear egress. Alternatively, a mature capsid, with multiple binding sites for the NEC that would create avidity effects, could provide a major driving force for the formation of an enveloping vesicle containing a coat composed of extended patches of NEC hexamers. Indeed, 100-nm patches of NEC coats form on the inner nuclear membrane in the vicinity of capsids (84). Hence, the NEC activity in infected cells is subject to both negative and positive regulation, but the exact regulatory mechanism remains to be elucidated.

DE-ENVELOPMENT

De-envelopment is a process in which perinuclear virions fuse with the outer nuclear membrane to release naked capsids into the cytosol (Figure 5). This stage of nuclear egress clearly requires additional viral proteins because it does not occur in NEC-transfected cells where perinuclear vesicles accumulate in herniations (79). Although the requirement for membrane fusion is implicit in de-envelopment, it must also include an uncoating step

whereby the stable NEC scaffold of the perinuclear vesicle must disassemble to liberate the naked capsid. Membrane fusion and uncoating could be coordinated or could occur sequentially. Finally, efficient nuclear egress would have to be unidirectional, favoring fusion of the perinuclear vesicles with the outer nuclear membrane over back fusion with the inner nuclear membrane. How this is accomplished is unknown, but one could speculate on the existence of a potential outer nuclear membrane receptor.

Nuclear Egress Complex Coat Disassembly

The hexagonal NEC scaffolds within the perinuclear vesicles are stable structures that need to be disassembled during de-envelopment. The US3 kinase in HSV-1 and PRV may also be involved in this process because, in its absence, these vesicles mostly accumulate in the perinuclear space in herniations (9–11), which is similar to what is seen in NEC-expressing transfected cells (79). HSV-1 US3 phosphorylates six serine residues within the N terminus of UL31 (103). Mutation of these serines to alanines results in the same phenotype as the lack of US3 kinase: retention of perinuclear vesicles in herniations (103). Phosphorylation of UL31 by the US3 kinase thus appears important for efficient de-envelopment even if not essential (103). Phosphorylation of the NEC may lead to structural rearrangements that disrupt NEC interactions within the hexagonal lattice and promote the disassembly of the NEC coat. US3 is, indeed, present in the perinuclear viral particles (9). By interfering with oligomerization, phosphorylation of the NEC could, in theory, both negatively control nuclear budding in the absence of the capsid and trigger disassembly of the NEC coat during de-envelopment, but this remains to be shown experimentally. Although deletion of US3 impairs nuclear egress, the resulting defect in viral growth is modest. This argues that US3, though important for efficient de-envelopment, is not essential.

Membrane Fusion During De-Envelopment

Membrane fusion, be it virus-cell or cell-cell, at a minimum requires attachment and membrane merging, the latter typically mediated by a protein catalyst termed a fusogen. Membrane fusion during cell entry by alphaherpesviruses, such as HSV and PRV, requires four viral glycoproteins gD, gB, and gH/gL plus a cellular gD receptor. According to the current model, gD is a receptor-binding protein, gB is the fusogen, and gH/gL regulates the function of gB by an unidentified mechanism (reviewed in 104). Glycoproteins gD, gB, and gH/gL are, indeed, present at the inner and outer nuclear membranes (105, 106) and in perinuclear particles (22, 107), albeit in their immature forms, raising the possibility that the entry glycoproteins may be involved in membrane fusion during de-envelopment. Glycoproteins gB and gH/gL have been reported as important for de-envelopment of several herpesviruses, but this requirement is not universal. Deletion of both gB and gH blocks efficient egress in HSV-1; yet, puzzlingly, deletion of either protein has little, if any, effect (106). Mutations in HSV-1 gB fusion loops impair nuclear egress, suggesting that its fusogenic activity is important in de-envelopment (108). However, it is unclear how either gB or gH/gL could suffice for membrane fusion during de-envelopment, although both are required for fusion during entry. Moreover, in the closely related PRV, neither gB nor gH appears necessary for efficient nuclear egress, and neither is even present in the perinuclear vesicles (109). In EBV, gB is necessary for nuclear egress (110), but in a related gammaherpesvirus, KSHV, it is not (111). Thus, whatever role gB or gH/gL has in de-

envelopment, they do not appear to function as conserved fusion machinery. In HSV, gB or gH/gL may instead be doing something else, perhaps docking the perinuclear vesicles at the outer nuclear membrane or transmitting the signal from the outside to the inside of the vesicle.

Moreover, although deletion of gB/gH/gL impairs nuclear egress, which is evident from the formation of dramatic herniations and a reduced number of cytoplasmic capsids, the resulting defect in viral growth is modest. Collectively, these observations suggest that some other fusion machinery, viral or cellular, is involved in de-envelopment. Among viral proteins, HSV-1 UL51 could play an important, albeit indirect, role in membrane fusion. In the absence of UL51, HSV-1 primary viral particles accumulate in the perinuclear space, indicating a role in de-envelopment, and viral titers drop 100-fold (112). UL51 is palmitoylated and localizes mainly to the Golgi. Treatment of PRV-infected cells with brefeldin A, which inhibits transport between the Golgi and the ER, shows a phenotype similar to that of a UL51 deletion: Primary virions accumulate in the perinuclear space and are unable to undergo de-envelopment (113). It is possible that, in the absence of UL51, trafficking of membrane fusogens required for de-envelopment is perturbed, which results in impaired de-envelopment. On the cellular side, nuclear ESCRT-III proteins and AAA-type ATPase p97, which control nuclear envelope reformation during telophase (99), are potential candidates.

Additional Viral and Cellular Proteins Implicated in Nuclear Egress, but Their Contributions Are Poorly Defined

A number of cellular and viral proteins have been reported to either bind NEC components or modulate nuclear egress in a species-specific manner, e.g., p32 (114, 115), emerin (114), endophilin A2 (116), Alix (117), UL21 (118), UL47 (119), and ICP22 (120), but none appear as essential to nuclear egress as UL31 and UL34, and none are required across herpesviruses. Nevertheless, these proteins may contribute to efficient nuclear egress in specific contexts: different herpesviruses, certain cell types, and so on. Although only UL31 and UL34 are universally required for nuclear egress, this complex multistep has a number of regulatory checkpoints that could be subject to different regulatory inputs. Future work needs to clarify the roles and the relative importance of each proposed protein for efficient nuclear egress.

NUCLEAR ESCAPE: BEYOND HERPESVIRUSES

Nuclear Budding in Uninfected Cells

Recent work has shown that the process of nuclear egress is not unique to herpesviruses and also occurs during the export of large cellular synaptic ribonucleoproteins (RNPs) in *Drosophila* (121). These RNPs are too large to exit the nucleus through nuclear pores and instead appear to undergo both budding into the inner nuclear membrane and de-envelopment at the outer nuclear membrane like herpesviruses. It is unclear how common the nuclear envelope budding is in host cells and whether it is restricted to certain cell types, for example, nondividing cells.

The existence of the nuclear egress pathway in uninfected cells, at first glance, suggests that herpesviruses may have hijacked this pathway. However, although torsins are essential for nuclear budding in uninfected cells, in their absence, vesicles formed in the perinuclear space remain attached to the inner nuclear membrane (122–124). The absence of torsin has only a modest effect on the efficiency of nuclear egress in HSV-1 (101). No cellular protein has yet been implicated in membrane fusion during the nuclear egress of large RNPs, but nuclear ESCRT complexes (99) are possible candidates. Whenever such proteins are identified, their involvement in herpesvirus nuclear egress should also be investigated. However, given that the NEC can vesiculate membranes without the help of other factors, one interesting possibility is that nuclear budding of RNPs may be driven by factors present in the large RNPs themselves. It is possible that an RNA rather than a protein could be such a factor, although no RNA has ever been suggested to deform a membrane. Further studies are clearly necessary to delineate the contributions of host factors to the nuclear egress of herpesviruses versus large RNPs and the mechanistic similarities and differences between these two processes.

Nuclear Budding Is Not Unique to Herpesviruses

Although herpesviruses are unique among animal viruses in undergoing nuclear budding, this process is also utilized by enveloped, dsDNA viruses of insects. In cells infected with baculoviruses *Autographa californica* nucleopolyhedrovirus and *Bombyx mori* polyhedrovirus, enveloped capsids are observed in the perinuclear space (125, 126). Whether these particles undergo de-envelopment to release capsids into the cytoplasm is unclear, although baculoviruses appear to undergo cytoplasmic maturation. The use of nuclear budding by baculoviruses is consistent with the large size of their capsids, 21 × 260 nm. Other insect viruses may also be able to bud into the nuclear membrane. Viruses that infect parasitic wasps and some ichnoviruses have been reported to bud through the inner nuclear membrane and then are released as extracellular virions surrounded by two unit membranes (127, 128). It appears that nuclear budding may be a more common nuclear escape strategy among viruses than we currently appreciate.

Nuclear Envelope Breakdown as Alternative Route for Nuclear Egress

Although the NEC is essential for nuclear egress and in its absence replication is strongly impaired, it is not abolished. Some capsids manage to escape into the cytosol, and mature virions are formed and released into the extracellular space. Thus, NEC-mediated nuclear egress represents the most efficient and preferred way for viral capsids to exit the nucleus, but other nuclear escape routes are possible.

Many viruses that replicate in the nucleus, e.g., adenoviruses, parvoviruses, and polyomaviruses, escape the nucleus by rupturing the nuclear envelope (129–131; reviewed in 2, 132). Although this is an acceptable strategy at late stages of viral replication, it is unfavorable early in viral infection. Herpesviruses predominantly use the envelopment/de-envelopment mechanism, but mutant PRV lacking UL31 or UL34 acquired the ability to initiate nuclear envelope breakdown (NEBD) upon multiple passages in culture (78, 133). PRV appeared to induce NEBD by manipulating mitosis-related processes (133). NEBD was also observed in HSV-1 in the absence of the cellular ATPase torsinA (134). Just as with

UL34-null PRV, viral titers were reduced. It can be assumed that any severe disruption in the normal nuclear egress pathway, regardless of the stage or the mechanism, forces the virus to exit by an alternative route.

Even though NEBD accomplishes the goal of releasing nuclear capsids into the cytoplasm, it eliminates an important checkpoint in capsid quality control. The abundance of defective capsids lacking viral DNA in the cytoplasm, which are capable of undergoing secondary envelopment and being released from the cell via the secretory pathway, results in high numbers of noninfectious viruses and thus in poor mutant viability. These observations point to NEC-mediated budding as an important quality-control mechanism, ensuring that primarily DNA-filled C capsids undergo maturation and are released from the cells.

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Glossary

HSV	herpes simplex virus
PRV	pseudorabies virus
LINC	linker of nucleoskeleton and cytoskeleton
SUN	Sad1p, UNC-84
KASH	Klarsicht, ANC-1, Syne homology
NEC	nuclear egress complex
HCMV	human cytomegalovirus
EBV	Epstein-Barr virus
KSHV	Kaposi’s sarcoma–associated herpesvirus
ESCRT	endosomal sorting complexes required for transport
RNP	ribonucleoprotein
NEBD	nuclear envelope breakdown

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SUMMARY POINTS

1. Herpesviruses replicate and encapsidate their dsDNA genomes in the nucleus yet undergo final maturation in the cytoplasm. Capsids are translocated from the nucleus into the cytoplasm by an unusual mechanism—termed nuclear egress—whereby capsids bud at the inner nuclear membrane (primary envelopment) and the resulting primary virions fuse with the outer nuclear membrane (de-envelopment). Cytoplasmic capsids undergo the second, and final, round of budding at cytosolic membranes to become mature infectious virions.
2. The NEC is conserved across the *Herpesviridae* family and consists of two proteins, UL31 and UL34, in alphaherpesviruses. The NEC localizes to the inner nuclear membrane and is essential for nuclear egress. It recruits kinases for lamina dissolution, recruits capsids to inner nuclear membrane for budding, and deforms the membrane around nucleocapsids. In vitro the NEC is sufficient for both membrane budding and scission of the nascent buds. The NEC drives budding by oligomerizing into a hexagonal lattice and scaffolding the inner surface of the bud.
3. All steps mediated by the NEC are likely positively and negatively regulated by a number of cellular and viral proteins in addition to posttranslational modifications. The precise details of how NEC activity is regulated are still unknown, but cellular and viral kinase activity appears important.
4. Perinuclear virions fuse with the outer nuclear membrane, a mechanism that is most likely not dependent on the viral glycoproteins that are needed for fusion during viral entry. The fusion mediators remain unknown and are likely host proteins. Viral kinase US3 may be involved in the disassembly of the NEC coat during de-envelopment.
5. The nuclear egress pathway is not unique to herpesviruses and is also utilized during export of large RNPs in insect cells. In addition, capsids of certain viruses that infect insects undergo budding at the inner nuclear membrane. Whether these mechanisms share common participants or features remains unclear.

FUTURE ISSUES

1. How is the budding activity of the NEC regulated during infection? How does the NEC select mature DNA-filled capsids for budding? How do capsids trigger NEC activity?
2. What mechanism ensures that perinuclear virions fuse with the outer nuclear membrane rather than with the inner nuclear membrane to increase the efficiency of nuclear egress? Is there a specific receptor on the outer nuclear membrane? What fusogen mediates fusion of the perinuclear virions with the outer nuclear membrane during de-envelopment?
3. Does the NEC coat disassemble during de-envelopment, and if so, how? What happens to the NEC after fusion?
4. How much do herpesviral and cellular nuclear egress have in common? Do common participants and mechanisms underlie phenotypic similarities or have the two systems evolved different solutions? Are nuclear ESCRTs recruited by herpesviruses to increase the efficiency of scission, or is the NEC alone sufficient for nuclear budding in infected cells?
5. Is nuclear egress limited to herpesviruses and dsDNA viruses of insects or is it more common than currently thought? Is it utilized by other animal viruses replicating in the nucleus, perhaps as an alternative, less destructive escape route?

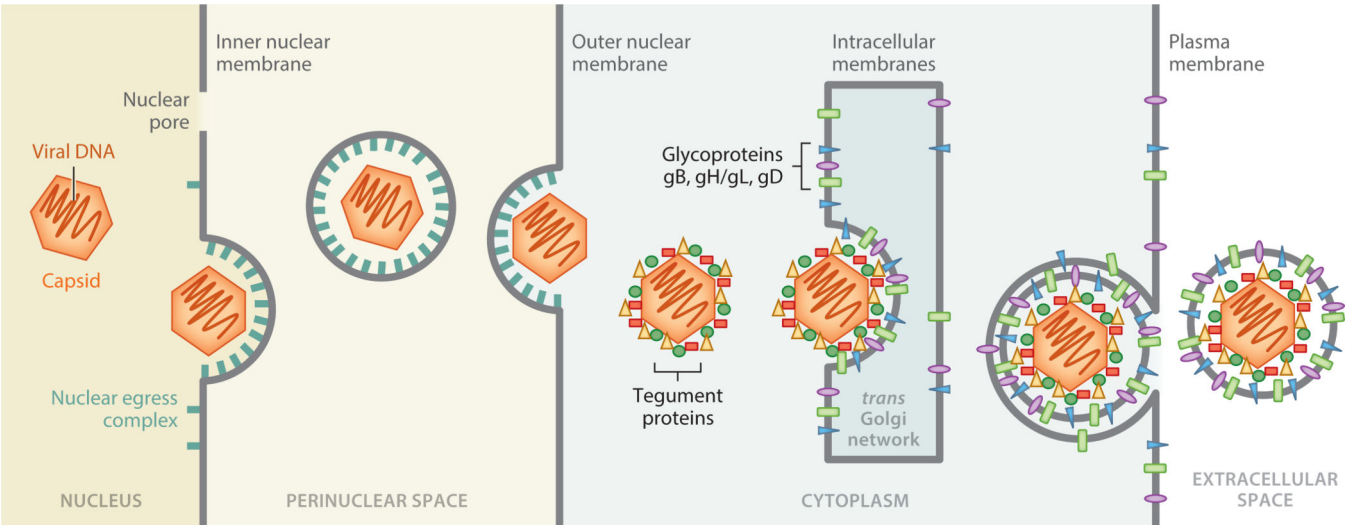

Figure 1.

Overview of herpesvirus egress. Viral nucleocapsids are assembled in the nucleus. To relocate into the cytoplasm, they bud at the inner nuclear membrane. This process is mediated by the nuclear egress complex. Resulting primary virions are translocated to the perinuclear space, where they fuse with the outer nuclear membrane. Naked nucleocapsids are released into the cytoplasm to undergo final maturation and acquire a tegument protein layer. Cytoplasmic capsids bud at membranes derived from the *trans* Golgi network or early endosomes and thereby obtain an envelope containing the glycoproteins needed for cell entry. Mature infectious virions hijack the secretory pathway to be released into the extracellular space.

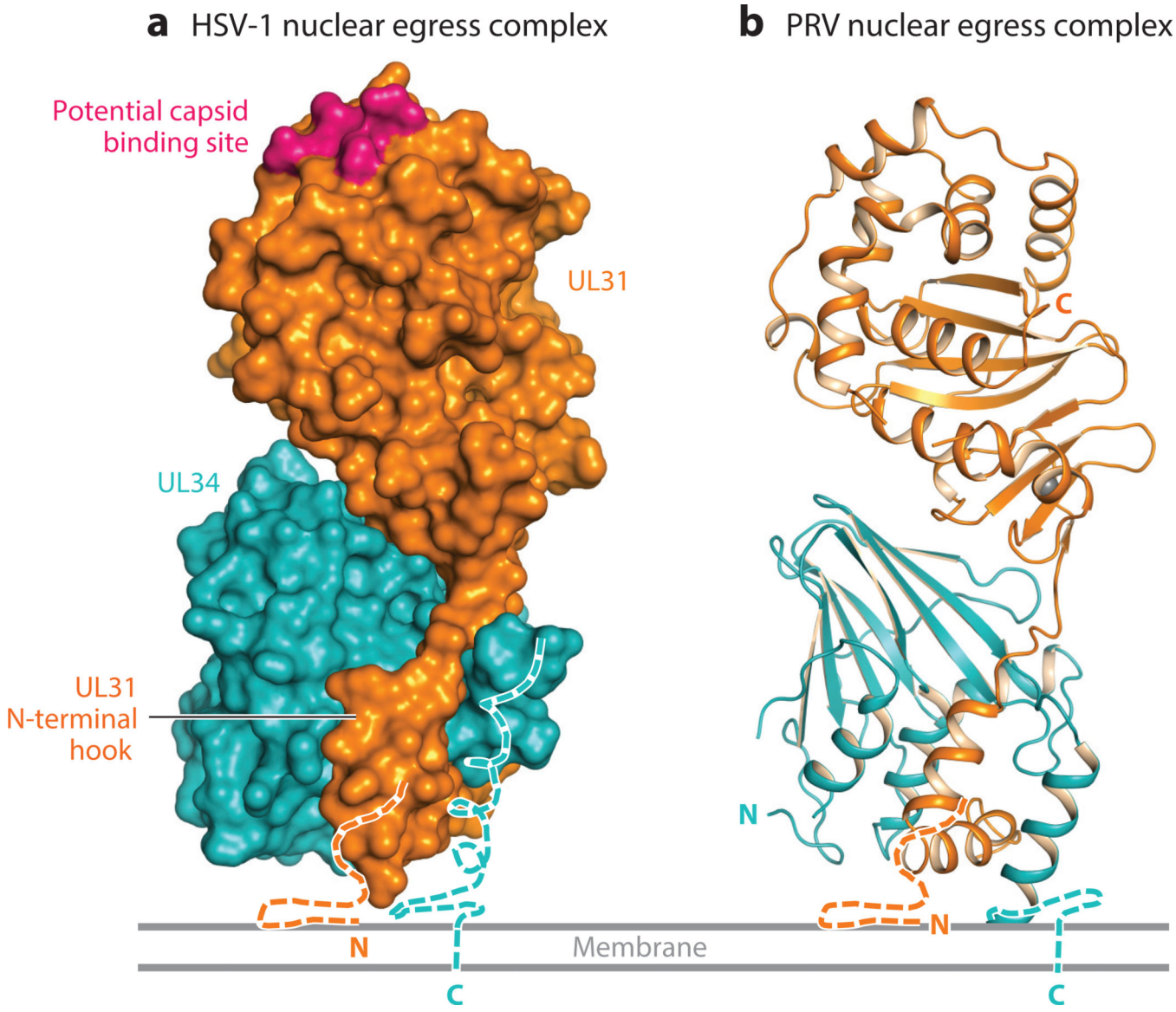

Figure 2.

Structural features of the nuclear egress complex of (*a*) herpes simplex virus 1 (HSV-1; PDB: 4ZXS) and (*b*) pseudorabies virus (PRV; PDB: 4Z3U). UL31 (*orange*) and UL34 (*teal*) form an elongated complex, with UL31 wrapping its N-terminal hook around UL34. The two molecules interact extensively, which implies high binding affinity. The membrane-proximal end is located at the bottom of the heterodimer in this orientation. The regions important for membrane interaction are missing from the structure and are indicated schematically, along with the membrane. The highly conserved surface patch on the membrane-distal end of UL31 is the putative capsid-binding site.

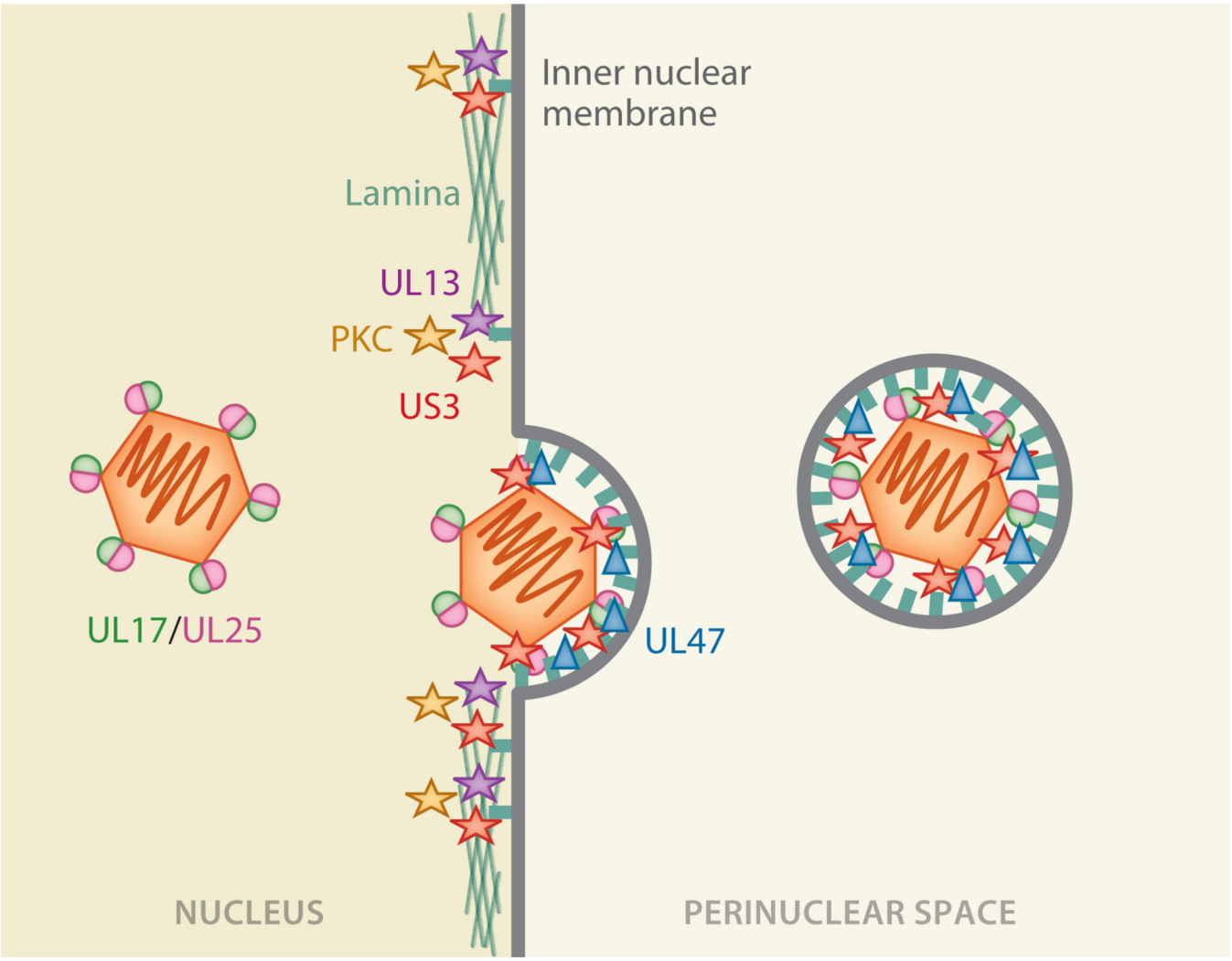

Figure 3.

Multiple proteins are involved in primary envelopment (nuclear budding). Although the nuclear egress complex can mediate vesicle budding by itself in vitro, this process appears subject to positive and negative regulation by a number of proteins during infection. Cellular and viral kinases (e.g., UL13, US3, and PKC) are recruited to the sites of primary envelopment for herpesviruses of all subfamilies. They phosphorylate proteins within the nuclear lamina as well as in the nuclear egress complex itself. This loosens the stiff lamina and allows capsids to be recruited to the inner nuclear membrane. Other viral proteins, such as UL47, have been implicated in regulating efficient nuclear egress, but their precise roles are still unclear. The nuclear egress complex is also responsible for capsid recruitment and may do so by binding the accessory capsid proteins UL17/UL25 or the major capsid protein VP5.

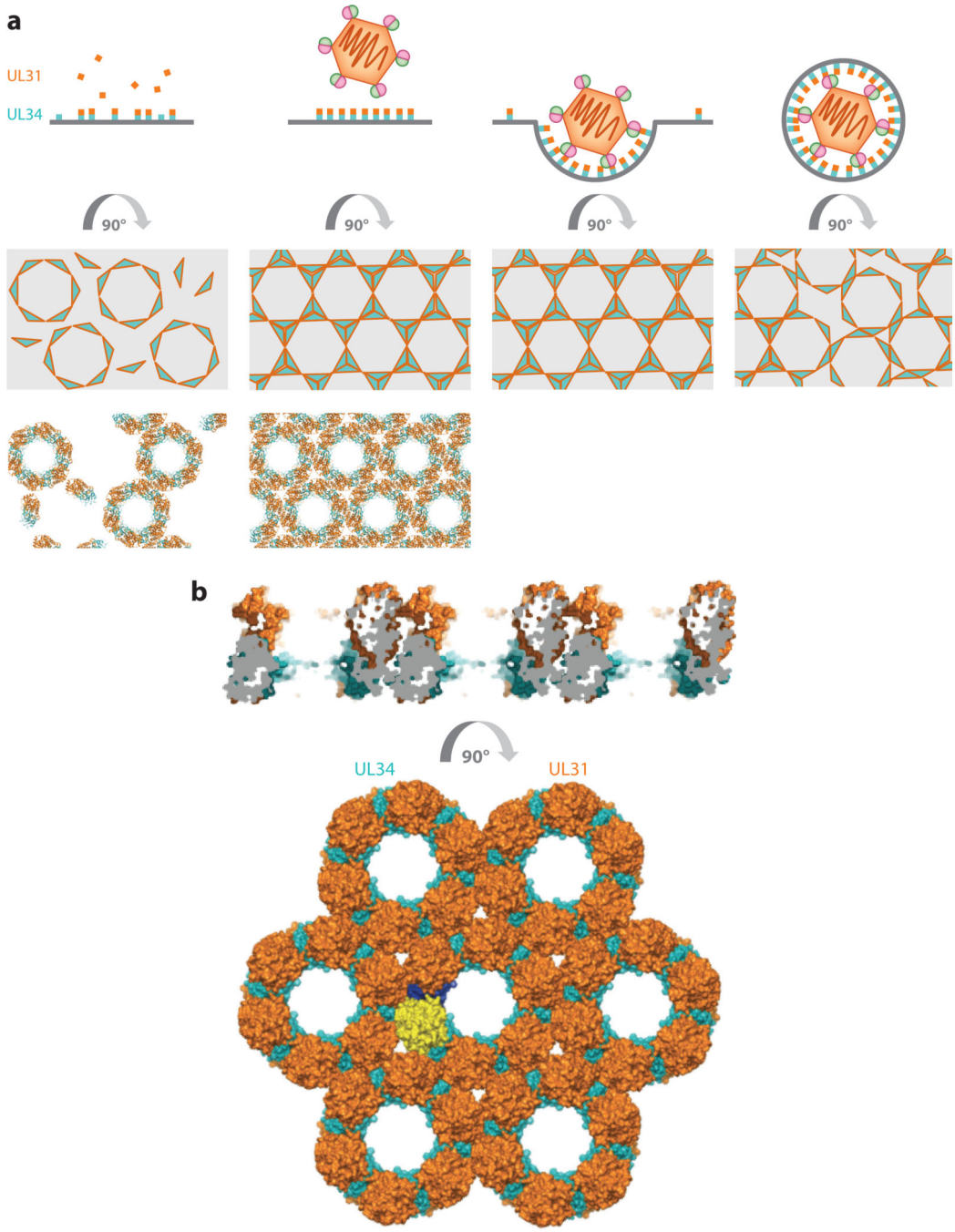

Figure 4.

Nuclear egress complex coat formation drives membrane deformation and budding. (*a*) UL34 (*teal*), anchored in the inner nuclear membrane, binds UL31 (*orange*) to form the nuclear egress complex heterodimer. The complex initially forms hexameric rings that eventually extend into a larger lattice when the capsid approaches. Conformational changes likely deform the membrane initially, but larger structural rearrangements are needed for sphere formation. This is possibly achieved by the introduction of errors within the hexagonal lattice. (*b*) The herpes simplex virus (HSV)-1 nuclear egress complex forms

crystalline hexagonal lattices (PDB: 4ZXS), which resemble the hexagonal coats observed by cryo–electron tomography. The detailed analysis of the lattice allowed the identification of regions important for coat formation and membrane budding. Figure adapted with permission from Reference 38.

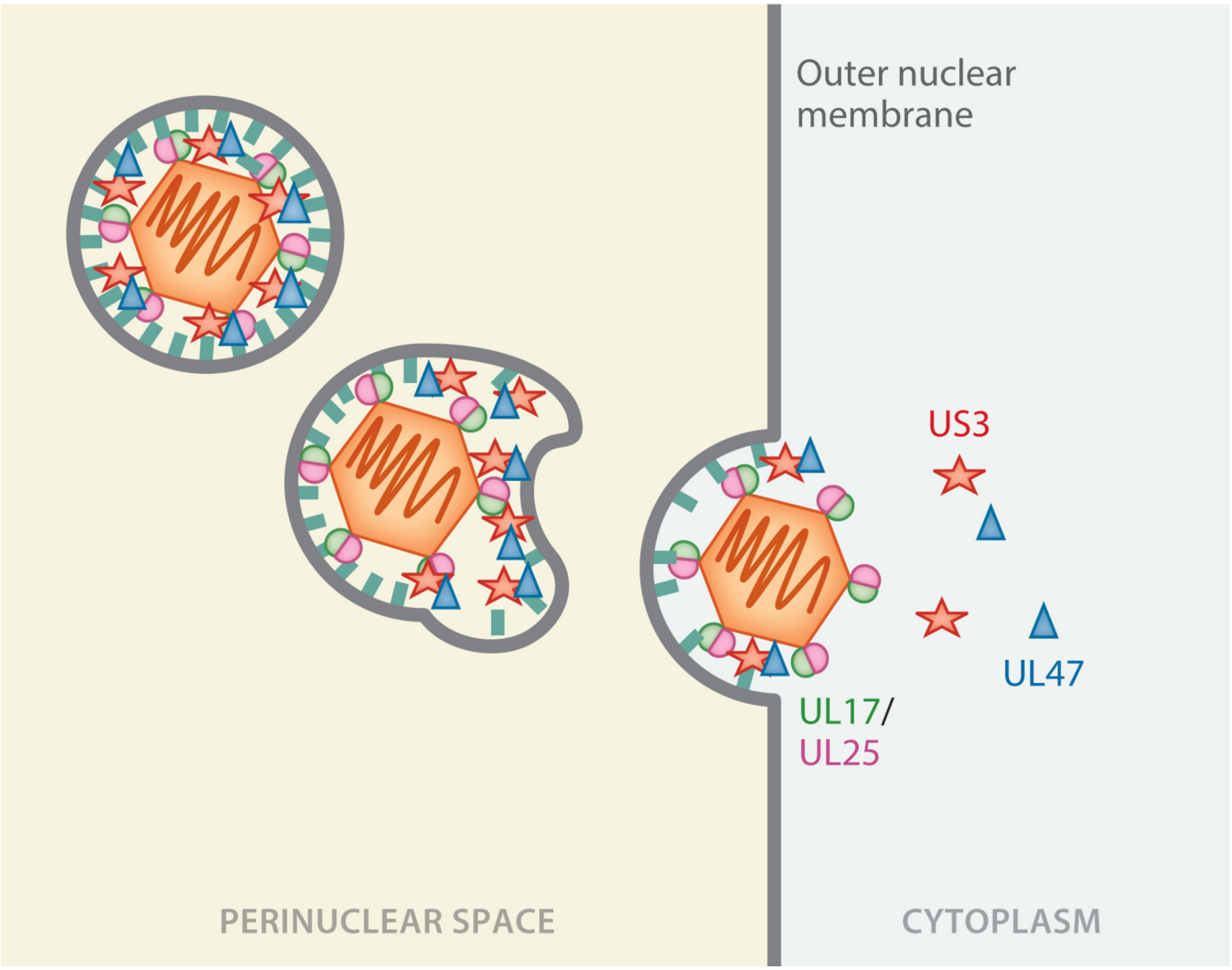

Figure 5.
De-envelopment at the outer nuclear membrane. It is still largely unclear how de-envelopment of herpesvirus capsids is mediated. Viral glycoproteins that are required for viral entry do not play the same role here, suggesting that an unidentified cellular fusion machinery drives the de-envelopment process. Some viral proteins, such as US3 and UL51, may be involved in the process, but their roles are unclear. US3 kinase activity may aid nuclear egress complex lattice disassembly prior or coincident with membrane fusion.