Selection of HSV capsids for envelopment involves interaction between capsid surface components pU_L31, pU_L17, and pU_L25

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During egress from the nucleus, HSV capsids that contain DNA (termed C capsids) are preferentially enveloped at the inner nuclear membrane over capsid types lacking DNA. Using coimmunoprecipitation and biochemical analyses of wild-type and mutant capsids, we identify an interaction between a complex of pU_L17/pU_L25, termed the C capsid-specific complex (CCSC), and pU_L31, a component of the nuclear egress complex (NEC). We also show that the interactions between these components are dependent on expression of all three proteins but occur independently of the pU_L31 interacting protein and NEC component pU_L34, as well as a kinase encoded by U_S3 that phosphorylates both pU_L31 and pU_L34. The interaction between the CCSC and pU_L31 in the NEC suggests a mechanism to conserve viral resources by promoting assembly of only those viral particles with the potential to become infectious.

virus assembly | virus egress

Herpesvirus nucleocapsids (type C capsids) are assembled in the nucleoplasm and are preferentially selected over other capsid types, such as type B that lack DNA, to undergo an initial or primary envelopment reaction at the inner nuclear membrane (INM) (reviewed in refs. 1 and 2). Primary envelopment requires the products of genes U_L31 and U_L34 in the HSV system (3–5). Orthologs of U_L31 and U_L34 are present in all known herpesviruses, and for those systems in which it has been studied, the requirement for these proteins in primary envelopment is conserved (6–9). U_L31 encodes a nucleoplasmic phosphoprotein, pU_L31 (10), that is maintained in close approximation to the INM by association with pU_L34, a type II integral membrane protein (5, 11–13). The bulk of pU_L34 is located within the nucleoplasm, with only five amino acids predicted to lie within the perinuclear space (14, 15). Although it has been established that pU_L31 and pU_L34 are incorporated into perinuclear virions (16, 17), whether the capsid engages the pU_L31/pU_L34 complex (also known as the nuclear envelopment complex or NEC) directly or indirectly is not known.

The U_L17 and U_L25 gene products interact, forming a stable complex (18). DNA-containing C capsids contain ≈75 copies of pU_L25, whereas B capsids contain ≈20 copies (10). Because of its enrichment in C capsids, the U_L25/U_L17 complex was named the C capsid-specific complex or CCSC (19). The CCSC bridges pentameric vertices to the adjacent 20 planar faces on the capsid surface (10, 19, 20). One hypothesis to explain how C capsids are selected for envelopment is that the products of U_L25 and U_L17 bind more efficiently to the surface of type C capsids after DNA packaging is complete (19), and these capsids subsequently engage the NEC complex either directly or indirectly. Consistent with this hypothesis is the observation that U_L17 and U_L25 null capsids do not become enveloped (21, 22). However, CCSC components have additional functions that may contribute indirectly to envelopment. For example, U_L17 is necessary for DNA to be cleaved and packaged (21), and U_L25 is required for optimal production of genomic termini and retention of cleaved DNA in the capsid (22–24). Thus, it is unclear whether lack of envelopment of pU_L17 or pU_L25 null capsids at the INM reflects the respective roles of these genes in assembly of DNA-containing capsids, or more directly as ligands to attach capsids to the NEC.

Supporting the latter possibility, the present study identifies an interaction between the U_L31 component of the NEC and CCSC components in infected cells. The data support a model in which the CCSC is added to capsids after DNA is inserted and engages pU_L31 either in the nucleoplasm or within the NEC at the INM to effectively select DNA-containing capsids for envelopment. Preferential envelopment of DNA-containing capsids is an elegant method to conserve cellular resources such that only capsids with the potential to produce infectious virions undergo subsequent steps in the virion assembly pathway.

Results and Discussion

Components of the CCSC Interact with pU_L31. Preliminary evidence suggested that the U_L31 proteins coimmunoprecipitated with pU_L25 and pU_L17 (Fig. S1). To investigate the pU_L17/pU_L25/pU_L31 interaction further, CV1 cells were mock infected or infected with viruses individually lacking the U_L17, U_L25, or U_L31 genes, and lysates of these cells were clarified, denatured, and subjected to gel electrophoresis. The electrophoretically separated proteins were then probed with antibodies to pU_L31, pU_L25, and pU_L17. This analysis showed (Fig. 1) that neither pU_L17, pU_L25, nor pU_L31 required any of the other proteins for accumulation in the clarified lysate. Moreover, antibody reactivity was specific, inasmuch as bands comigrating with each protein were greatly reduced or not detected in lanes containing lysates of cells infected with the respective null virus (e.g., the pU_L17-specific band was not detected in lysates of cells infected with the U_L17 null virus).

Clarified cellular lysates were also subjected to immunoprecipitation with pU_L31- and pU_L17-specific antibodies. Immunoprecipitated material was separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies to pU_L17, pU_L25, and pU_L31. As shown in Fig. 1, both pU_L31- and pU_L17-specific antibodies immunoprecipitated or coimmunoprecipitated pU_L31, pU_L17, and pU_L25 from lysates of cells infected with wild-type virus HSV-1(F). Moreover, loss of expression of any one protein precluded coimmunoprecipitation of the others. Specifically, whereas the pU_L17-specific antibody immunoprecipitated pU_L17 from lysates of cells infected with the U_L31 and U_L25 null viruses, pU_L31 was not coimmunoprecipitated with this antibody from lysates of cells infected with the U_L25 null mutant, and pU_L25 was not coimmunoprecipitated

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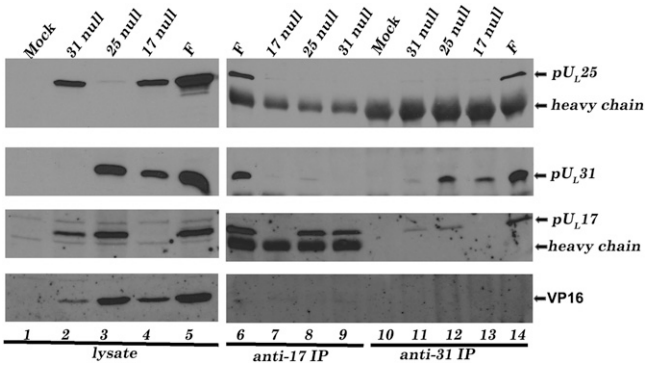

Fig. 1. Coimmunoprecipitation of pU_L17, pU_L25, and pU_L31 requires expression of all three proteins. CV1 cells were infected with HSV-1(F), U_L17 null, U_L25 null, or U_L31 null viruses, or were mock infected. At 18 h after infection cells were lysed, and soluble lysates (*Left*) or immunoprecipitation reactions (*Right*) using anti-pU_L17 or anti-pU_L31 antibodies were electrophoretically separated and immunoblotted with anti-pU_L25, anti-U_L31, anti-pU_L17, or anti-VP16 antibodies.

from lysates of cells infected with the U_L31 mutant. Similar results were obtained using pU_L31-specific antibody: whereas this antibody efficiently immunoprecipitated pU_L31 from lysates of cells infected with wild-type virus or the U_L25 or U_L17 deletion viruses, pU_L17 was not coimmunoprecipitated as efficiently with this antibody from lysates of U_L25 null infected cells (Fig. 1, lane 12). Moreover, pU_L25 did not coimmunoprecipitate with pU_L31 from lysates of cells infected with the U_L17 deletion mutant that were reacted with the U_L31-specific antibody (Fig. 1, lane 13).

The following was observed in control reactions. (*i*) Faint bands comigrating with pU_L31-specific bands were occasionally detected in electrophoretically separated anti-pU_L31 immunoprecipitations from mock and U_L31 null virus-infected cell lysates (Fig. 1, lanes 10 and 11, respectively), but these bands were much less prominent than pU_L31 immunoprecipitated from lysates of cells infected with HSV-1(F), U_L17 null, or U_L25 null viruses. Moreover, these faint bands were inconsistent in size and intensity from experiment to experiment (for example, Fig. S1, lane 6, *Bottom*). (*ii*) Bands attributable to pU_L25 were not detected in immunoprecipitations from lysates of U_L31 null virus-infected cells reacted with the U_L31 antibody, nor from lysates of U_L17 null virus-infected cells reacted with the pU_L17-specific antibody. (*iii*) Some pU_L17 immunoreactivity was observed in immunoprecipitation reactions from U_L31 null virus-infected cells reacted with pU_L31 specific antibody, suggesting that some pU_L17 was immunoprecipitated nonspecifically (Fig. 1, lane 11). Nevertheless, intensity of this band was lower than was detected in similar reactions from HSV-1(F) or U_L25 null virus-infected cell lysates and was inconsistent from experiment to experiment (for example, Fig. S1, lane 6, *Middle*). (*iv*) Immunoblotting electrophoretically separated immunoprecipitated material with antibody to VP16, an abundant HSV-1 protein, indicated that this protein was not coimmunoprecipitated with anti-pU_L31 or anti-pU_L17 antibodies despite readily detectable amounts in the various cellular lysates.

Given the observation (Fig. 1, lanes 6 and 14) that pU_L31 associated with pU_L17 and pU_L25, which constitute the CCSC, we next asked whether pU_L31 associated with capsids. Capsids were purified from lysates of cells infected with wild-type HSV-1 (F) on sucrose gradients using standard protocols. Fractions from the gradients were collected, and protein in each fraction was trichloroacetic acid (TCA) precipitated, denatured, electrophoretically separated, transferred to nitrocellulose, and probed with antibodies to VP5 (the major capsid protein), pU_L25, pU_L17, or pU_L31. The results are shown in Fig. 2*A*. Protein-specific signals were quantified by densitometry, and the percentage of immunoreactivity in each fraction relative to total immunoreactivity with the same antibody is shown in Fig. 2*B*.

pU_L31 was detected in most fractions to varying extents but accumulated in two peaks, a smaller peak in fraction 5 and 6 and a second peak containing lighter material with maximal levels in fractions 10 and 11. A minor VP5 peak corresponding to the presence of C capsids was observed in fraction 6. A second, more intense VP5 peak corresponding to B capsids was observed with maximum immunoreactivity in fraction 10. Fractions containing material less dense than capsids (fractions 13–19) also contained substantial amounts of pU_L31 immunoreactivity, with amounts decreasing in each fraction as they approached the top of the gradient. Although overall immunoreactivity was lower with the pU_L25-specific antibody, fractions that contained pU_L31 also contained pU_L25. pU_L25 immunoreactivity peaked in fractions 6 and 10–11, thus corresponding to C and B capsids, respectively. High levels of pU_L17 also associated with capsid-containing fractions. However, peak amounts of pU_L17 accumulated in fractions slightly less dense than capsids (with a maximal amount in fraction 13). These data suggest that pU_L31 associates with wild-type capsids but also pU_L17 and pU_L25 that are free of capsids.

To determine whether the capsid association of pU_L31 was dependent on pU_L25, capsids were isolated from lysates of cells infected with a U_L25 deletion mutant and purified on a continuous sucrose gradient. Proteins within the fractions were precipitated with TCA and analyzed on immunoblots probed with VP5 and pU_L31. As shown in Fig. 3*A*, the absence of pU_L25 greatly affected the distribution of pU_L31 in the gradient. As opposed to gradients containing wild-type capsids (Fig. 2*A*), pU_L31 was not detected to a great extent in any fraction from U_L25 deletion mutant-infected cell lysates, although there was barely detectable immunoreactivity in fraction 10. As a control, a small amount of U_L25 deletion mutant-infected cell lysate on the same immunoblot was probed with pU_L31 antibody and revealed ample immunoreactivity (Fig. 3*A*, lane labeled lysate). We conclude that pU_L31 association with capsid-containing fractions requires pU_L25.

Because pU_L17 interacts with pU_L25, efforts were made to determine the contribution of pU_L17 to pU_L31 capsid association. Capsids were therefore purified from cells infected with the U_L17 deletion virus (this virus only produces B capsids), and immunoblots of gradient fractions were probed with VP5- and pU_L31-specific antibodies. As shown in Fig. 3*B*, the absence of pU_L17 did not eliminate association of pU_L31 with capsid-containing fractions. On the other hand, considerably less pU_L31-specific immunoreactivity was observed in fractions containing material lighter than capsids than was apparent upon similar analyses of lysates infected with wild-type virus. We conclude that pU_L17 is dispensable for association of pU_L31 with capsids but augments association of pU_L31 with material less dense than capsids.

Efforts were then expended to characterize the material other than capsids with which pU_L31 associated. Because the known interacting partner of pU_L31 is the integral membrane protein pU_L34, and this would conceivably tether pU_L31 to membranes, capsids were purified from lysates of cells infected with the U_L34 deletion mutant and immunoblotted with the U_L31-specific antibody. As shown in Fig. 3*C*, the absence of U_L34 caused increased pU_L31 immunoreactivity in the gradient relative to other capsid preparations. Two peaks of pU_L31 immunoreactivity were observed, with maximal levels in fractions 6–8 and 12–14 in the particular gradient shown in Fig. 3*C*. However, substantial pU_L31 immunoreactivity was also detected near the top of the gradient. We attribute the prominence of the peak in fractions 6–8 relative to other capsid preparations to the enrichment of

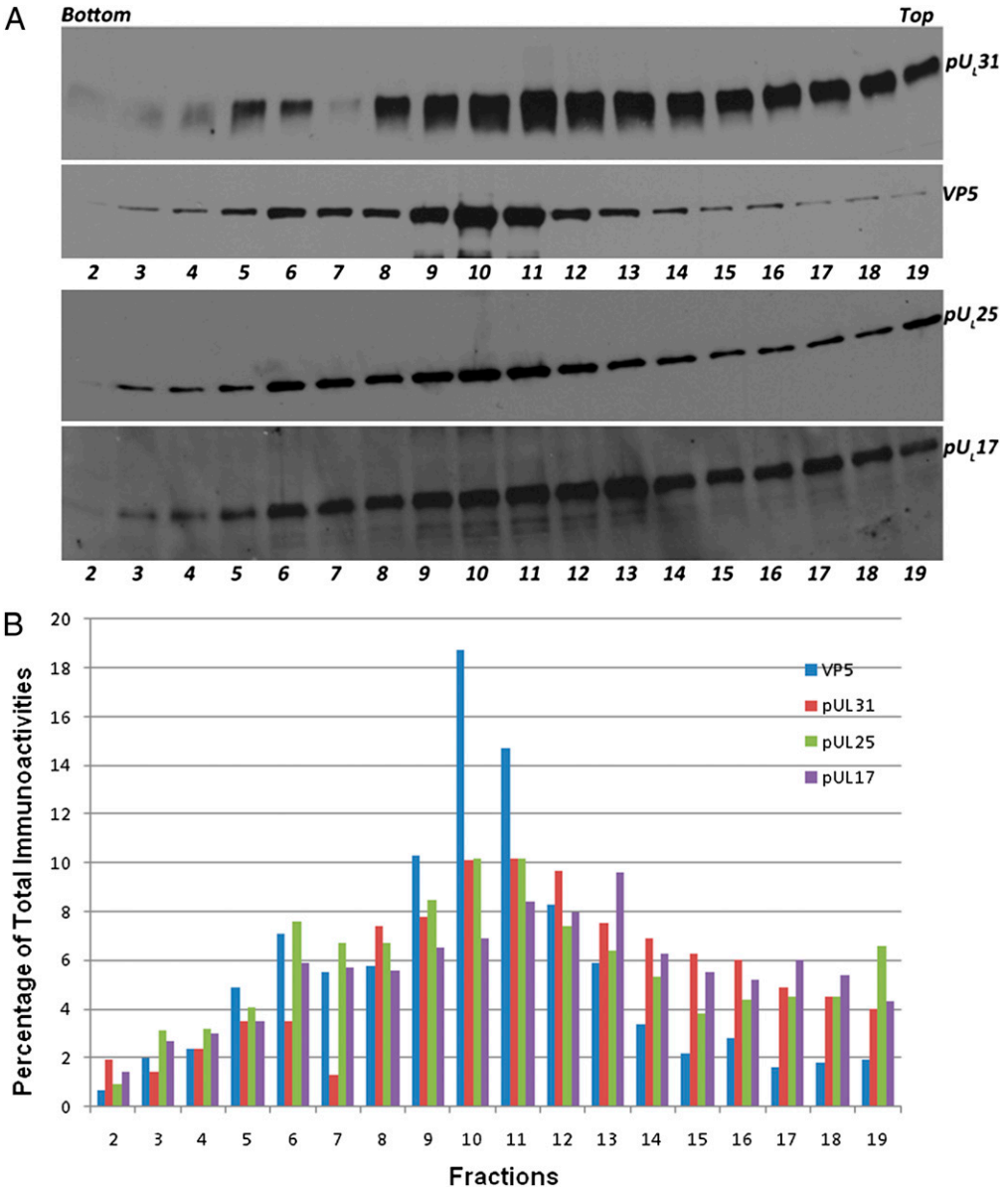

Fig. 2. Immunoblot of sucrose gradient-fractionated wild-type capsids probed with anti-pU$_L$31, anti-pU$_L$25, or anti-VP5 or anti-pU$_L$17 antibodies. CV1 cells were infected with HSV-1(F) at a multiplicity of infection of 5 pfu/cell. At 20 h after infection cells were collected and lysed. Capsids were pelleted by centrifugation through a sucrose cushion and were then resuspended and separated on a continuous sucrose gradient (*Materials and Methods*). Approximately 0.5-mL fractions as determined by eye were collected from the bottom of the gradient (fraction 1) to the top (fraction 20) using a Buchler Auto Bensi-Flow IIC gradient collector. Proteins in fractions were TCA precipitated, and pellets were denatured and solubilized in SDS. Fractions 2 through 19 were separated on an SDS polyacrylamide gel and analyzed by immunoblotting, followed by reaction with appropriate conjugates, application of chemiluminescence substrate, exposure to X-ray film, and digital scanning. (*A*) *Top* and *Upper Middle:* Images of the same blot first probed with pU$_L$31 then stripped and reprobed with VP5 antibodies. *Lower Middle* and *Bottom:* A second blot containing identical samples probed with pU$_L$25 antibody. Immunoreactivity was then stripped, and the blot was probed with pU$_L$17 antibody. (*B*) Percentage of immunoreactivity of a given antibody in different fractions as quantified by Image J software.

C capsids in nuclei of cells infected with the U$_L$34 null mutant, inasmuch as these capsids do not become enveloped and therefore do not exit the nucleoplasm. VP5 also accumulated in two peaks, with maximal amounts in fractions 7 and 11. Thus, although substantial pU$_L$31 immunoreactivity was contained in capsid-containing fractions, considerable amounts were detected in fractions containing material less dense than capsids. These results confirm the association of pU$_L$31 with capsids and indicate that capsid association and association with material lighter than capsids occurred independently of pU$_L$34.

Another possibility was that the components less dense than capsids include remnants of the nuclear lamina because pU$_L$31 has been shown to interact with lamin A, a major lamina component (25). Alternatively, this material may represent association with virion tegument proteins, inasmuch as pU$_L$17 has been shown to interact not only with pU$_L$25 but also major tegument components pU$_L$46 and pU$_L$47 (26). To address these possibilities, we probed immunoblots identical to those above with antibodies to either lamin A or pU$_L$47. Despite some nonspecific background staining with pU$_L$47 antibody in most fractions, discrete bands attributable to either pU$_L$47 or lamin A/C were not detected in the gradient (Fig. S2), even though such bands were readily detected in the total cellular lysate. These data suggest that the less dense material lacking capsids but con-

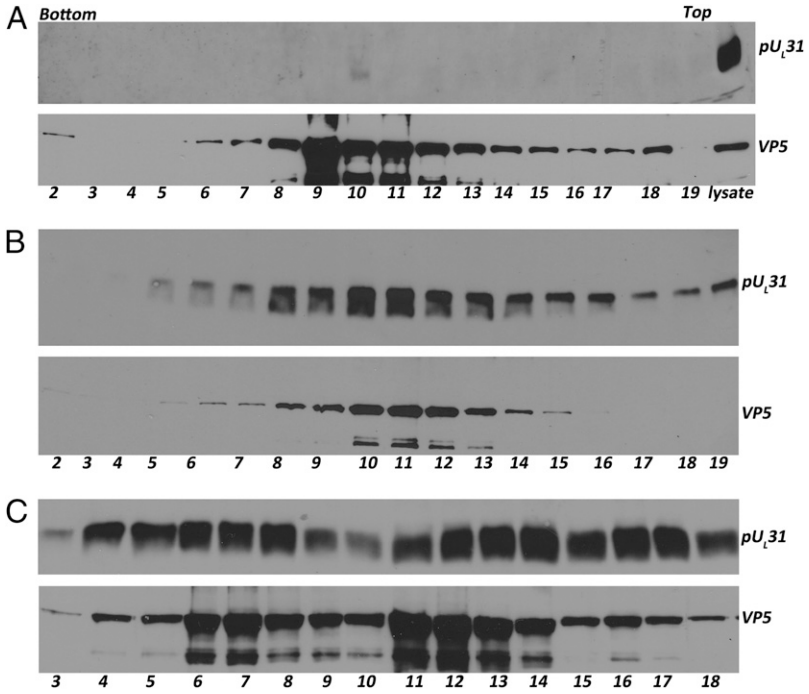

Fig. 3. pU$_L$31 capsid association requires pU$_L$25 but not pU$_L$17 nor pU$_L$34. CV1 cells were infected with 5.0 pfu/cell of (*A*) U$_L$25 null, (*B*) U$_L$17 null, or (*C*) U$_L$34 null viruses. Cellular lysates prepared at 20 h after infection were pelleted through a sucrose cushion. Material in the pellets was resuspended and separated by ultracentrifugation through 10 mL continuous sucrose gradients. Twenty ≈0.5-mL fractions were collected from bottom to top of each gradient. Material in fractions was TCA precipitated and solubilized in SDS. Fractions 2–19 (*A* and *B*) from the U$_L$25 null and U$_L$17 null gradients and fractions 2–18 (*C*) of the U$_L$34 null gradient were denatured in SDS, electrophoretically separated, and subjected to immunoblotting with VP5 and pU$_L$31 specific antibodies.

taining pU$_L$17, pU$_L$25, and pU$_L$31 did not contain abundant tegument proteins or nuclear lamina components.

Given the observations that pU$_L$31 associated with capsids, the observed coimmunoprecipitation of pU$_L$17, pU$_L$25, and pU$_L$31 (Fig. 1) might simply reflect immunoprecipitation of the entire capsid with pU$_L$17- or pU$_L$31-specific antibodies. To rule out this possibility, cells were infected with a virus lacking U$_L$18, which encodes VP23. Because VP23 is an essential component of capsid triplexes that link adjacent capsomeres, its absence precludes capsid formation (27–29). As shown in Fig. 4, pU$_L$25 was coimmunoprecipitated efficiently with pU$_L$31 antibody from lysates of cells infected with the U$_L$18 deletion virus and from wild-type virus HSV-1(F), indicating that pU$_L$31 and pU$_L$25 could interact even in the absence of intact capsids. On the other hand, less pU$_L$17 was immunoprecipitated with the pU$_L$31-specific antibody from lysates of cells infected with the U$_L$18 deletion virus compared with levels from HSV-1(F)–infected cell lysates. Thus, either the presence of capsids or VP23 enhanced the coimmunoprecipitation of pU$_L$17 with pU$_L$31 antibody. To distinguish between these possibilities, we reacted U$_L$18 null virus-infected cell lysates with pU$_L$17-specific antibody and found that this antibody efficiently coimmunoprecipitated both pU$_L$25 and pU$_L$31. Taken together, these data indicate that pU$_L$17, pU$_L$25, and pU$_L$31 can interact even in the absence of intact capsids.

We were also interested to determine whether pU$_S$3, a kinase that phosphorylates pU$_L$31 and thereby regulates virus egress from the nucleus (30), and pU$_L$34, the integral membrane protein that normally interacts with pU$_L$31 in the NEC, affected the pU$_L$31/pU$_L$17/pU$_L$25 interaction. As shown in Fig. 4, neither the absence of U$_S$3 or of pU$_L$34 precluded or even greatly affected coimmunoprecipitation of pU$_L$31, pU$_L$17, or pU$_L$25 with antibodies to either pU$_L$31 or pU$_L$17.

The data in this work indicate an interaction between pU$_L$31, a component of the NEC, and pU$_L$25 and pU$_L$17, constituting the CCSC. Evidence supporting the interaction comes from the observations that (*i*) coimmunoprecipitation of any two of the three proteins requires expression of all three; (*ii*) coimmunoprecipitation of pU$_L$17, pU$_L$25, and pU$_L$31 can occur independently of capsid formation and pU$_L$34, the interaction partner of pU$_L$31; and (*iii*) that capsid association of pU$_L$31 requires pU$_L$25. It follows that pU$_L$31 on the capsid would localize coincident with the CCSC [i.e., at vertices linking hexons and pentons (19, 20)].

The data presented herein suggest a model in which enrichment of pU$_L$17 and pU$_L$25 on C capsids helps select these capsids for envelopment by interacting with pU$_L$31 of the NEC, eventually leading to capsid budding at the nuclear membrane. Data supporting this model include the observations that capsids do not exit the nuclei of cells infected with U$_L$17 and U$_L$25 null mutants, pU$_L$25 is more abundant on DNA-containing capsids as opposed to other capsid types, and pU$_L$31 is required for optimal envelopment of capsids at the INM (4, 5, 10, 21, 22, 31, 32).

U$_L$25 null capsids of pseudorabies virus (PRV), a well-studied swine herpesvirus, accumulate against the nuclear membrane of infected cells, suggesting they are able to attach to the INM (33). Thus, components of the capsid other than pU$_L$25 are sufficient to mediate INM tethering in this system. It follows that PRV U$_L$25 is required to trigger capsid budding at the INM, perhaps by inducing a conformational change in the budding machinery,

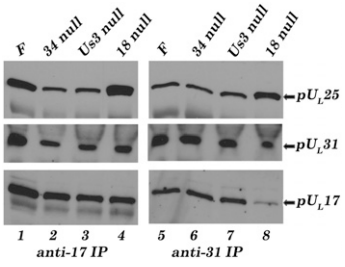

Fig. 4. The pU$_L$17/pU$_L$25/pU$_L$31 complex forms in the absence of pU$_L$18, intact capsids, pU$_L$34, or pU$_S$3. CV1 cells were infected with HSV-1(F), U$_L$18 null, U$_L$34 null, or U$_S$3 null viruses. At 18 h after infection cells were lysed and immunoprecipitated with anti-pU$_L$17 (*Left*) or anti-pU$_L$31 (*Right*) antibodies. The immunoprecipitated material was then subjected to immunoblotting with anti-pU$_L$25, anti-U$_L$31, or anti-pU$_L$17 antibodies.

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which likely includes the NEC. Supporting this role of the NEC is the observation that expression of PRV pU_L31 and pU_L34 in the absence of other viral proteins and capsids is sufficient to induce budding from the INM (34). In the HSV-1 system, pU_L25-null capsids were not observed to attach excessively to the INM (22), but whether HSV-1 pU_L25 is truly dispensable for attaching to the INM of HSV-infected cells requires further investigation.

The sequence of events governing U_L31 association with the CCSC is not entirely clear. Any model must take into account previous observations that pU_L31 can associate with pU_L34 in the absence of other viral proteins (5, 35). Three possibilities differ in the timing of addition of pU_L31 to capsids. Specifically, (*i*) the capsid lacking pU_L31 migrates to the NEC, and the CCSC-NEC engagement occurs exclusively at the INM; (*ii*) pU_L31 associates with capsid-bound CCSC in the nucleoplasm, to eventually bind to pU_L34 or a complete NEC in the INM; or (*iii*) pU_L31 associates with capsid-free pU_L17/pU_L25, and the three proteins eventually interact with capsids, followed by interaction between the pU_L31 moeity and pU_L34 at the INM.

Although the first of these possibilities was favored by us primarily because pU_L31 accumulates at the nuclear membrane of infected cells (5), data in the present study suggest this model may be too simplistic. This conclusion is based on observations indicating that capsid-free pU_L17/pU_L25 can associate with pU_L31. Specifically, pU_L31 coimmunoprecipitates with CCSC components in the absence of pU_L34 and capsids (Fig. 4), is required for optimal pU_L25 and pU_L17 coimmunoprecipitation (Fig. 1), and can associate with capsids independently of pU_L34 (Fig. 3). Moreover, pU_L17, pU_L25, and pU_L31 accumulate in fractions of sucrose gradients containing material less dense than capsids (Fig. 2), suggesting that these proteins can form a capsid-free complex. These putative complexes (i.e., in fractions containing material less dense than capsids) are dependent on pU_L25, reduced in lysates of cells infected with a U_L17 null virus, do not contain lamin A/C or the pU_L17-interacting protein pU_L47, and are increased in the absence of pU_L34, presumably because more pU_L31 is present in the nucleoplasm of these cells and is therefore more available for interaction with the capsid rather than with pU_L34 at the INM (5). Thus, although pU_L31's migration in the sucrose gradient required pU_L25 and was influenced by pU_L17, association of pU_L31 with the nuclear membrane or nuclear lamina was not relevant to this migration in the gradient. Caveats include the possibilities that these complexes represent capsid fragments released after cellular lysis or that some of the deletion mutant phenotypes reflect off-pathway effects. Further work will be necessary to determine the sequence of events involved in the NEC-CCSC interaction.

An interesting possibility is that consequential to higher pU_L25/pU_L17 occupancy, DNA-containing C capsids are more likely to bind pU_L31, which in turn may bind the NEC. Thus, pU_L31 on C capsids helps mediate their selection for envelopment at the INM to conserve resources such as sections of nuclear membrane embedded with viral glycoproteins and associated tegument proteins for envelopment and egress. Although selection of DNA-containing capsids for budding may conserve resources, it should be noted that the selection process is not foolproof, inasmuch as enveloped capsids lacking DNA can be observed occasionally in the perinuclear space of cells infected with HSV by electron microscopy. This may reflect the association of some pU_L25 and pU_L17 with capsids lacking DNA (Fig. 2 and refs. 10, 36, and 37). Some of these capsids lacking DNA become decorated with pU_L31 (Fig. 2), making them competent to interact with pU_L34 at the INM, which drives their attachment and occasional envelopment. Further work will be needed to determine the stoichiometry of pU_L31 in different capsid types and under different conditions. An important caveat for such quantification is that the interaction between pU_L31 and the CSSC is ultimately transient, inasmuch as it must be disrupted at the deenvelopment step in the egress pathway (Fig. 5, step 4). These observations suggest tight regulation of the interaction. Phosphorylation of pU_L31 by the U_S3 viral kinase may represent one regulatory mechanism because this has been shown to promote virion egress from the perinuclear space (30).

Materials and Methods

Cells and Viruses. CV1 and Vero cells were obtained from the American Type Culture Collection and were propagated in DMEM supplemented with 10% newborn calf serum and antibiotics as described previously (38, 39). HSV-1 F strain [HSV-1(F)] and R7027 (U_S3 null) were described previously and propagated in Vero cells (38, 39). Mutant viruses U_L17 null, U_L34 null, V3161 (U_L31 null), KUL25 NS (U_L25 null), U_L18 null, and the complementing cell lines used to propagate them (CV1-17, R1310, UL31-CV1, C8-1, and G5, respectively) have been described previously (3, 4, 21, 27, 31, 37, 40). All of the mutant viruses were derived from HSV-1(F), except U_L25 null virus KUL25 NS, which was derived from the HSV-1 strain KOS.

Sucrose Gradient Sedimentation and Capsid Purification. Approximately 1.6×10^9 CV1 cells from two 850-cm ^{2} roller bottles were infected with HSV-1(F), U_L17 null, U_L25 null, or U_L34 null viruses at a multiplicity of infection of 5 pfu/cell. At 20 h after infection, cells were collected and washed with PBS. The cell pellets were lysed in 25 mL of lysis buffer [20 mM Tris·Cl (pH 7.6), 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, and protease inhibitor], sonicated, and the lysates were precleared by centrifugation for 15 min at $11,400 \times g$. The supernatants were then subjected to ultracentrifugation at $104,000 \times g$ for 1 h in a Beckman SW28 rotor through a 5.0-mL 35% (wt/vol) sucrose cushion prepared in TNE buffer [500 mM NaCl, 20 mM Tris (pH 7.6), and 1 mM EDTA]. The pellets containing capsids were resuspended in 300 μL of TNE by brief sonication on ice, layered on a 20–50% sucrose gradient, and centrifuged at $108,000 \times g$ for 1 h in a Beckman SW41 rotor. Twenty fractions were collected from the gradients by eye from the bottom of the tube to the top using a Buchler Auto Densiflow IIC fraction collector. The fractions were precipitated by the addition of TCA to 200 mg/mL and incubation at 4 °C overnight, and pelleted by centrifugation at $13,400 \times g$ for 10 min in

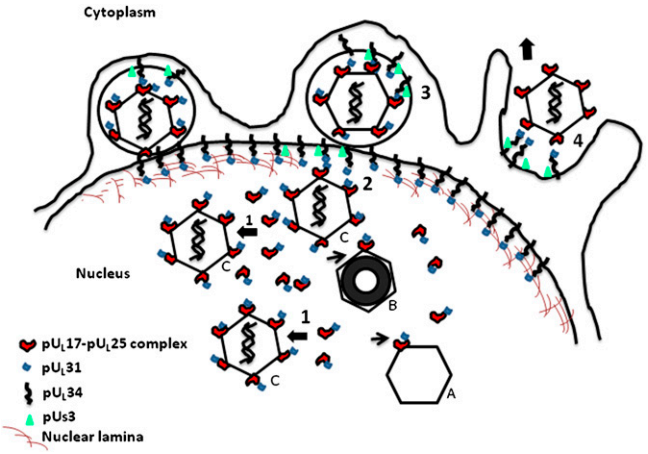

Fig. 5. Model of nuclear capsid egress. Icosahedral C capsids contain DNA, B capsids lack DNA but contain an internal proteinaceous scaffold (black circle), and A capsids lack internal contents. The nuclear lamina is perforated by the action of cellular and viral kinases (such as viral U_S3) to allow nucleocapsids access to the INM. pU_L17 and pU_L25 interact with one another and with pU_L31 in the nucleoplasm. The $pU_L17/pU_L25/pU_L31$ complex then attaches to capsids (step 1). This complex is present on all capsid types but enriched on the surface of C capsids. The pU_L31 moiety in this complex then interacts with pU_L34 at the INM or a complete pU_L31/pU_L34 complex (the nuclear envelopment complex or NEC; step 2). The larger number of $pU_L17/pU_L25/pU_L31$ complexes on C capsids may recruit locally high concentrations of NECs to trigger envelopment (step 3). During envelopment, the viral kinase pU_S3 is also incorporated into the perinuclear virion. Deenvelopment (step 4) is triggered by pU_S3-mediated phosphorylation of pU_L31 and viral glycoprotein gB (gB is not diagrammed). pU_L31 is retained in association with pU_L34 at the outer nuclear membrane, whereas the capsid is released into the cytosol for eventual budding at cytoplasmic membranes.

a microfuge. The pellets were washed once with cold acetone, resuspended and boiled in SDS sample buffer, and proteins therein were separated on 10% polyacrylamide SDS gels and transferred electrically to nitrocellulose membranes for immunoblotting.

Immunoprecipitation. Approximately 8×10^6 CV1 cells were infected with 5 pfu of various viruses per cell. Cells were collected at 18 h after infection, pelleted by centrifugation, and lysed by resuspension in 800 μL of immunoprecipitation buffer [1% Nonidet P-40, 20 mM Tris (pH 7.4), 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin]. After clarification at 16,000 × *g* for 10 min in a microfuge, the supernatants were incubated with primary antibodies and Gamma Bind G Sepharose 4B beads (GE Healthcare) overnight at 4 °C with rotation. For immunoprecipitation with anti-pU$_L$17 antibody, rabbit anti-chicken Ig Y was added to the primary antibodies and clarified lysates before addition of the Gamma bind G beads as previously described (18). The beads with bound proteins were pelleted and washed 4 times with ice-cold immunoprecipitation buffer, and protein was eluted from the beads in 2× SDS/PAGE buffer [100 mM Tris·HCl (pH 6.8), 4.0% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM fresh DTT], separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes for immunoblotting.

Immunoblotting. The procedure was described previously (41). Primary antibodies were diluted in PBS containing 2% BSA. Primary antibodies were added to immunoblots for 2 h at room temperature or overnight at 4 °C at the following dilutions: chicken anti-pU$_L$17 1:2,000 (37), rabbit anti-pU$_L$31 1:1,000 (5), mouse anti-pU$_L$25 monoclonal antibody 4A11 E4 1:1,000 (20), mouse anti-VP5 monoclonal antibody 1:1,000 (H1.4, BioDesign), rabbit anti-VP13/14 (pU$_L$47) 1:1,000 (26), goat anti-VP16 (Santa Cruz Biotechnology, SC-1728) 1:500, and anti-lamin A/C mouse monoclonal antibody 1:200 (Santa Cruz Biotechnology, SC-7292). The bound immunoglobulins were detected by reaction with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG or anti-chicken IgY and visualized by enhanced chemiluminescence (Thermo Scientific) followed by exposure to X-ray film. In some experiments, the blot was stripped by incubating in buffer containing 62.5 mM Tris·HCl (pH 6.8), 2% SDS, and 100 mM B-mercaptoethanol at 50 °C for 30 min. Stripped blots were washed extensively, blocked, and reprobed by immunoblotting as described above. Chemiluminescent signals of individual bands were quantified with Image J software.

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