Packaging Determinants in the UL11 Tegument Protein of Herpes Simplex Virus Type 1

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The UL11 gene of herpes simplex virus type 1 encodes a 96-amino-acid tegument protein that is myristylated, palmitoylated, and phosphorylated and is found on the cytoplasmic faces of nuclear, Golgi apparatus-derived, and plasma membranes of infected cells. Although this protein is thought to play a role in virus budding, its specific function is unknown. Purified virions were found to contain ~700 copies of the UL11 protein per particle, making it an abundant component of the tegument. Moreover, comparisons of cell-associated and virion-associated UL11 showed that packaging is selective for underphosphorylated forms, as has been reported for several other tegument proteins. Although the mechanism by which UL11 is packaged is unknown, previous studies have identified several sequence motifs in the protein that are important for membrane binding, intracellular trafficking, and interaction with UL16, another tegument protein. To ascertain whether any of these motifs are needed for packaging, a transfection/infection-based assay was used in which mutant forms of the protein must compete with the wild type. In this assay, the entire C-terminal half of UL11 was found to be dispensable. In the N-terminal half, the sites of myristylation and palmitoylation, which enable membrane-binding and Golgi apparatus-specific targeting, were found to be essential for efficient packaging. The acidic cluster motif, which is not needed for Golgi apparatus-specific targeting but is involved in recycling the protein from the plasma membrane and for the interaction with UL16, was found to be essential, too. Thus, something other than mere localization of UL11 to Golgi apparatus-derived membranes is needed for packaging. The critical factor is unlikely to be the interaction with UL16 because other mutants that fail to bind this protein (due to removal of the dileucine-like motif or substitutions with foreign acidic clusters) were efficiently packaged. Collectively, these results suggest that UL11 packaging is not driven by a passive mechanism but instead requires trafficking through a specific pathway.

Herpes simplex virus type 1 (HSV-1) contains more than 40 different proteins, and these are partitioned into three compartments: the nucleocapsid, the surrounding but poorly defined tegument layer, and the glycoprotein-containing lipid envelope. Although the mechanisms by which the virion components come together to produce an infectious particle are not understood, the overall morphogenesis pathway is well defined (32). In brief, DNA-containing capsids are assembled in the nucleus and then transported into the cytoplasm by budding and fusion events at the inner and outer nuclear membranes, respectively. The cytoplasmic nucleocapsids are subsequently transported to Golgi apparatus-derived membranes, where the viral glycoproteins accumulate and maturation budding takes place. Tegument proteins are thought to be added to the nucleocapsid in an orderly fashion at various steps along the transport pathway and during budding. Vesicles containing the mature virions subsequently fuse with the plasma membrane, enabling release into the extracellular medium.

One of the smallest HSV-1 components is the lipid modified, 96-amino-acid tegument protein, UL11 (Fig. 1). All herpesviruses encode a homolog of this protein (32), but in all cases, the function is unknown. Viruses that fail to express their UL11 homolog invariably exhibit defects in maturation budding and accumulate nucleocapsids in the cytoplasm (2, 10, 22, 23, 26, 27, 48, 51, 52); hence, it is possible that this protein is a component of the budding machinery. However, it is also possible that it serves indirectly (e.g., perhaps by recruiting components of the actual machinery to the site of budding).

UL11 is synthesized on free ribosomes and is cotranslationally modified with myristate, a 14-carbon fatty acid that enables binding of proteins to the cytoplasmic faces of cellular membranes (11, 26, 29, 42, 45). In infected cells, it accumulates on nuclear and Golgi apparatus-derived membranes (1), but when expressed in the absence of all other viral proteins, it is found primarily on the Golgi apparatus (7). Recent studies have shown that UL11 has at least four sequence motifs that influence its subcellular localization, all of which map to the first half of the protein and are found in the homologs from other herpesviruses (Fig. 1). In addition to the N-terminal myristylation site, which enables the protein to bind weakly and nonspecifically to membranes (11, 29, 42), there are three nearby cysteines, at least one of which is posttranslationally modified with palmitate, a 16-carbon fatty acid that enables tight membrane binding and Golgi apparatus accumulation (24, 45). A dileucine-like (LI) motif and an acidic cluster (DIESEEEE) enable UL11 to be recycled from post-Golgi apparatus locations, and in the absence either of these, higher levels of the protein accumulate on the plasma membrane (7, 24, 25). The LI and DIESEEEE are also needed for binding to UL16 (25, 56), another poorly-understood tegument protein (3, 37, 41). Moreover, mutants in which the DIESEEE sequence is replaced with foreign acidic clusters (those from

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Nef or furin) fail to bind UL16 even though they are capable of recycling (25).

The goal of the experiments described here was to ascertain which features of UL11 are needed for virion packaging. Because the critical 5’ half of the U11 gene overlaps with U12, the product of which is essential for transport of capsids from the nucleus (50, 60), it was not feasible to construct recombinant viruses carrying the mutations of interest. For the same reason, previously described “null” mutants (2, 27) were not of use because they actually express large N-terminal fragments of UL11, which would complicate the interpretation of packaging studies. Therefore, the experiments below made use of a transfection/infection-based assay in which mutant forms of UL11 must compete with the wild type (wt) for packaging. The results suggest that packaging is not driven by a passive mechanism but instead requires transport of UL11 through a specific pathway.

MATERIALS AND METHODS

Cells and viruses. Vero and A7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum, penicillin (250 U/ml), and streptomycin (131 μg/ml). Cells infected with the KOS strain of HSV-1 (53) were grown in DMEM supplemented with 2% fetal bovine serum, 25 mM HEPES buffer, glutamine (0.3 μg/ml), penicillin, and streptomycin.

Antibodies. UL11-specific antiserum was derived from rabbits and has been described previously (25). Purified VP16- and GFP-specific antibodies (derived from rabbits) were obtained from Clontech (product no. 3844-1 and 8367-1, respectively).

UL11 constructs. All of the green fluorescent protein (GFP)-tagged UL11 derivatives used in this study have been described previously (24, 25). To produce constructs that can have a different C-terminal tag, a small sequence encoding an epitope from hemagglutinin (HA [YPYDVPDYA]) followed by a stop codon from rabbits) were obtained from Clontech (product no. 3844-1 and 8367-1, respectively).

Vero cells were transfected with plasmids encoding GFP-tagged UL11. The goal of the experiments described here was to ascertain which features of UL11 are needed for virion packaging. Because the critical 5’ half of the U11 gene overlaps with U12, the product of which is essential for transport of capsids from the nucleus (50, 60), it was not feasible to construct recombinant viruses carrying the mutations of interest. For the same reason, previously described “null” mutants (2, 27) were not of use because they actually express large N-terminal fragments of UL11, which would complicate the interpretation of packaging studies. Therefore, the experiments below made use of a transfection/infection-based assay in which mutant forms of UL11 must compete with the wild type (wt) for packaging. The results suggest that packaging is not driven by a passive mechanism but instead requires transport of UL11 through a specific pathway.

RESULTS

Characterization of UL11 within extracellular virions. Two attributes of UL11 that are relevant to its packaging are the number of copies and the number of differentially modified species present in the virion. The number of UL11 molecules in radiolabeled, purified virions was determined by comparing it to proteins of known copy number (Fig. 2 and Table 1). Using VP5 (960 copies) and VP16 (1,647 copies) as standards (38, 39, 49, 62), it was estimated that approximately 700 copies of UL11 are packaged per particle, making it an abundant component of the tegument.

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aged with a subsequent alteration taking place to eliminate the upper species.

A previous report has demonstrated that UL11 is phosphorylated when expressed in transfected cells as a fusion protein with GFP (24). To determine whether virally encoded UL11 is also modified and whether the three species represent different phosphorylated states of the protein, immunoprecipitated proteins from infected-cell lysates were treated with protein phosphatase in the presence or absence of inhibitors. Treatment with the enzyme resulted in 65% and 30% decreases in the upper and middle species of UL11, respectively, and a 100% increase in the fastest-migrating species (Fig. 3B and Table 2). The addition of inhibitors abrogated the observed changes in band intensities, suggesting that phosphatase itself, and not the treatment conditions, was responsible for the effects. These data suggest that the two slower-migrating species are phosphorylated forms of UL11, whereas the fastest-migrating species is unmodified. Attempts to confirm these findings by labeling with $^{32}$P were unsuccessful due to difficulties in resolving the three $^{32}$P-labeled UL11 species (data not shown).

Taken together, these data suggest that the most highly phosphorylated form of UL11 is not found within virions. This finding is similar to what has been recently reported with the cytomegalovirus homolog of UL11, pp28 (20).

**Development of the UL11 packaging assay.** While it would be of interest to study the packaging of UL11 mutants in the complete absence of the wild-type protein, overlap of the first 30% of UL11 gene with the essential UL12 gene impedes the construction of recombinant viruses that carry the mutant alleles of interest. For the same reason, all previously reported UL11-“null” mutants still express substantial portions of the protein (2, 27), and it is not known what effects these would have on packaging of trans-expressed UL11. Therefore, to ascertain whether any of the sequence motifs in UL11 are required for packaging, a transfection/infection-based assay was developed in which mutant constructs must compete with the wild-type, virally encoded protein (see Materials and Meth-

**TABLE 1. Average number of UL11 molecules per virion**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Avg no. of molecules relative to a VP16</th>
<th>VP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>845</td>
<td>NDb</td>
</tr>
<tr>
<td>2</td>
<td>538</td>
<td>584</td>
</tr>
<tr>
<td>3</td>
<td>688</td>
<td>727</td>
</tr>
</tbody>
</table>

a As standards, VP16 contains a total of 1,647 copies per virion (62) and VP5 contains a total of 960 copies per virion (49).

b ND, not done.

**TABLE 2. Phosphatase treatment of UL11 species**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Radioactivity in UL11 band$^c$:</th>
<th>Total counts$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper</td>
<td>Middle</td>
</tr>
<tr>
<td>Mock</td>
<td>33 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>PPase</td>
<td>12 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>PPase + inhibitor</td>
<td>25 ± 3</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

$^c$ Relative position of the gel bands within the UL11 triplet. The percentage of radioactivity found in each band was calculated, and the average of results from four independent experiments ± standard deviation is shown.

$^d$ Combined counts for the three UL11 species averaged from the four experiments.
ods). In brief, Vero cells were transfected with various UL11-fusion constructs and subsequently infected with HSV-1. Relative levels of protein present in the cell lysates and extracellular virions were then quantitated by Western blotting with antisera specific for the tag (to detect trans-expressed UL11 constructs) or VP16 (as a harvesting and loading control). Preliminary experiments revealed that trans-expressed UL11 is indeed competitive for virion packaging, and the efficiency was the same whether the C-terminal tag was GFP or an epitope from HA (data not shown). Packaging was found to increase proportionally with increased levels of expression (Fig. 4A); however, densitometry measurements revealed that at low levels, the Western blot assay ceased to be linear. Therefore, all of the UL11-GFP derivatives used in this study were titrated to express at high levels and in equal amounts (data not shown). Moreover, antibodies specific for UL11 were not used since alterations in the short (96 amino acids) UL11 sequence seemed likely to eliminate epitopes and adversely affect the assay.

**Subcellular localization of UL11-GFP constructs in transfected/infected cells.** The eight constructs used in this study were wt UL11, UL11.Δ51–96, Myr(−)UL11, UL11.CCC(−), UL11.ACT(−), UL11.nfAC, UL11.furAC, and UL11.L18A/I19A (Fig. 1). Because these were previously characterized in cells that expressed no other viral proteins (24), it was important to ascertain whether their subcellular locations are altered in the context of an infection. To address this, transfected Vero cells were infected with HSV-1 and subsequently visualized by confocal microscopy. For the most part, all of the constructs exhibited the expected patterns (Fig. 5) (data not shown). For instance, wt UL11-GFP construct was set at 100% packaging efficiency. Shown is the average of four independent experiments.
but not transfected cells (Fig. 5) (data not shown). This nuclear targeting, which has been observed previously by means of immunofluorescence in fixed cells (1), was abolished by removing either the acidic cluster or dileucine motif, suggesting that these sequences provide UL11 with nuclear targeting specificity in Vero cells, presumably through interactions with other viral proteins.

**Packaging of UL11 mutants.** The C-terminal half of UL11 has been found to be dispensable for all known properties of the protein, including membrane binding, intracellular trafficking, and interaction with UL16 (7, 24, 25). To determine whether it is also dispensable for virion incorporation, the N-terminal half of UL11 was fused to GFP (UL11.D51–96) and examined in the packaging assay. Unlike GFP, which was not detected within extracellular virions, UL11.D51–96 was packaged to almost the same level as wt UL11 (Fig. 4B and C). This finding suggests that all information required for packaging is contained within the first half of the protein. The small reduction observed with UL11.D51–96 is likely the result of having GFP positioned much closer to the packaging signals, but this hypothesis was not tested further.

The N-terminal half of UL11 contains two sites that are modified with fatty acids, and these work together to enable binding to Golgi apparatus-derived membranes. Cotranslational addition of myristate enables weak but nonspecific binding to membranes, while the subsequent addition of palmitate (following the initial membrane-binding event) enables accumulation in a perinuclear compartment (24). While it seemed likely that proper targeting would be needed for packaging, it was possible that interactions with another viral protein (e.g., UL16) would enable UL11 to be packaged in a membrane-independent manner. To address this, mutants defective for one or both modifications [UL11.CCC(–) and Myr(–).UL11, respectively] were tested, and these failed to be packaged in an efficient manner (Fig. 6A and B). Although this suggests that UL11 must be on Golgi apparatus-derived membranes to be packaged efficiently, a small but reproducible amount of Myr(–).UL11 was incorporated at a level above that seen with GFP.

The N-terminal half of UL11 also contains acidic cluster and dileucine-like (LI) motifs (Fig. 1), both of which are required for plasma membrane-to-Golgi apparatus trafficking and the interaction with UL16 (7, 24, 25). Nevertheless, mutants lacking either of these signals [UL11.AC(–) and UL11.L18A/I19A, respectively] still accumulate at the perinuclear compartment in transfected (24, 25) and transfected/infected cells (Fig. 5). Thus, if localization to this compartment is sufficient to enable passive packaging of UL11, then constructs lacking either motif should still be packaged like wt UL11-GFP. This was not found to be the case. The LI mutant actually exhibited enhanced packaging in most experiments, while the acidic cluster mutant was highly defective (Fig. 7A and B). Packaging was restored by inserting the well-characterized acidic clusters from furin (57) or human immunodeficiency virus type 1 (HIV-1) Nef (43) in place of the native motif (mutants UL11.furAC and UL11.nefAC, respectively), thus providing gain-of-function evidence for a role of these motifs in packaging. The ability of UL11.furAC, UL11.nefAC, and UL11.L18A/I19A to be packaged efficiently despite their inability to bind to UL16 (25) suggests that interactions between these two tegument proteins are not required for packaging.
DISCUSSION

The experiments described here address the packaging mechanism for the UL11 protein, a component of the tegument that is thought to play a role in virus budding. Because of overlap with the upstream U12 gene, which encodes the essential alkaline nuclease, the construction and characterization of mutant viruses were not feasible, and instead, a transfection/infection assay was used in which variants of UL11 must compete with the virus-encoded, wild-type protein.

Limitations of the transfection/infection assay. The mixed population of UL11 species inherent in the assay raises at least three unanswered questions. First, does wild-type UL11 assist in the packaging of any of the trans-expressed mutants or is it truly just a competitor? So far, no evidence for interactions among UL11 molecules has been observed in GST-UL11 pull-down experiments (25) or in yeast two-hybrid assays (56), but a multimerization domain could await discovery. Second, does trans-expressed UL11-GFP reduce the packaging of virus-encoded UL11 (i.e., is there a maximum of ~700 molecules of UL11 permitted per virion)? This is a difficult question to answer because most virions produced in the assay come from untransfected Vero cells, and these create a large background that obscures the analysis of the small virion population that contains UL11-GFP. One way to address this would be to isolate and infect cell lines that express different levels of UL11-GFP. Third, do any of the mutants have a dominant-negative effect on virus assembly and release? Again, the large background of virions produced by untransfected cells makes this question difficult to address, but it needs to be kept in mind for those mutants that exhibit reduced levels of packaging. In spite of these uncertainties, the assay has provided much information regarding the mechanism of UL11 packaging.

Requirements for UL11 packaging. All of the information required for packaging is present within the first half of UL11. This region contains determinants for membrane binding, intracellular trafficking, and interaction with UL16 (7, 24, 25). Whether the second half of the protein is entirely dispensable for infectivity remains to be seen. Previously constructed “null” mutants still express the first ~30% of the protein and exhibit a 1,000-fold reduction in the release of extracellular virions (2, 27). Thus, it will be of interest to learn whether infectivity is normal when only the second half of the gene is missing and is further reduced when a true null mutant is created.

Multiple sequence motifs within the first half of UL11 were found to have functions in packaging. N-terminal myristylation is required for membrane binding (7), and not surprisingly, this modification was also found to be critically important for packaging. Nevertheless, a small, yet reproducible, fraction of Myr(−)UL11 was found in extracellular virions. This is likely the result of an interaction with other viral components (e.g., capsid-associated UL16), rather than a nonspecific incorporation resulting from overexpression, because GFP-only molecules were not packaged when produced at the same level.

Palmitylation was also found to be important for UL11 packaging. This modification adds a 16-carbon, saturated fatty acid to cysteine residues, usually near the N or C termini of target proteins (45). Similar to various cellular and viral proteins, including UL51 of HSV-1 (40), palmitylation of UL11 is required for proper targeting to Golgi apparatus-derived membranes (24). Thus, the reduced packaging of UL11.CCC(−) is most likely the result of not accumulating at the site of virus budding. Interestingly, palmitylation has also been found to be important for the targeting of many cellular (e.g., CD4, G proteins, and Src family kinases) and viral (e.g., HIV-1 Gag) proteins to detergent-resistant, lipid-raft domains within various membrane compartments (4, 17, 30, 34, 59). In addition to serving as sites for signal transduction events, these locations are thought to function as platforms for virus assembly and budding (12). Although HSV-1 has not been reported to assemble on detergent-resistant, lipid-raft domains, it is possible that palmitylation enables UL11 to target specifically to raft domains at, or en route to, the site of budding. Studies are under way to determine whether or not UL11 associates with rafts when expressed by itself or with other virion components.

The most striking findings from this study were seen when the membrane-trafficking motifs in the first half of UL11 were altered. In particular, packaging failed with the acidic cluster deletion mutant but was enhanced with the LI mutant. These results are particularly interesting because both mutants accumulate at a perinuclear location that resembles that for the wild type. Although it is possible that the acidic cluster mutant has a structural defect that merely precludes packaging, the ability of foreign acidic clusters of different sequences (furAC) and/or lengths (nefAC) to restore virion incorporation argues against that hypothesis. Moreover, loss of the interaction with UL16 does not explain the phenotype because constructs that have foreign acidic clusters or lack the LI motif are readily packaged even though they also are unable to participate in this interaction (24, 25).

Because acidic clusters and LI motifs are known to be involved in membrane trafficking (6, 21, 47, 58), it is possible is that UL11 must traverse a particular pathway, perhaps by way of the plasma membrane, as a prerequisite to packaging. This pathway may enable the formation of essential protein-protein interactions or posttranslational modifications. In this model, UL11 has two available pathways it can enter, depending upon which trafficking signal is recognized by the sorting machinery. Recognition of the acidic cluster would lead to virion packaging, while recognition of the LI motif would serve another purpose. Hence, loss of the acidic cluster would block packaging and loss of the LI would enhance it by eliminating the competing pathway.

Precidence for dual-pathway trafficking is provided by the HIV-1 Nef protein, which has several similarities to UL11. Nef is small (205 residues), is peripherally bound to membranes via myristate (but not palmitate), has dileucine and acidic cluster motifs for trafficking between the trans-Golgi network and the plasma membrane, and has an affinity for lipid rafts (15). Unlike UL11, it is quite clear why Nef travels to the plasma membrane: to downregulate cellular surface proteins, most notably CD4 (the receptor for HIV-1) and major histocompatibility complex class I (MHC-I [needed for immune surveillance]). The mechanisms by which these two tasks are accomplished are different (15). After binding CD4, the dileucine motif of Nef links the complex to clathrin-mediated endocytic machinery (18, 19, 46), thereby sending the proteins down a pathway for degradation in lysosomes. In contrast, after binding MHC-I, the acidic cluster is recognized by PACS-1, a protein that in turn links the complex to clathrin adaptors and
results in downregulation to the trans-Golgi network (5, 13, 43, 58). With this in mind, it is easy to imagine that UL11 might have a role in downregulating viral or host proteins from the cell surface, with one pathway leading to the site of maturation budding.

Although the experiments described here represent the first packaging analyses for a UL11 homologue, the importance of endocytosis for the packaging of other herpesvirus proteins has been considered in a variety of studies. Some of these have shown that endocytosed proteins can be packaged, but this route is not essential. A few examples of this are provided by HSV-1 gB (14) and pseudorabies virus gE and Us9 (8, 9). However, there are other examples of viral proteins for which endocytosis appears to be required for packaging. Examples of this are gE from varicella-zoster virus (28, 33) and gB from human cytomegalovirus, for which an acidic cluster motif is required (44, 55). Further studies of the trafficking pathways used by herpesvirus proteins are needed to provide mechanistic explanations for these differences.

**Phosphorylation states of UL11.** Although the proteins of the tegument generally exist in multiple phosphorylated forms, evidence provided here and elsewhere shows that only the underphosphorylated species are found in purified virions (16, 20, 31). Combined with data showing that phosphorylation promotes dissociation of tegument proteins following entry, a model has been proposed in which HSV-1 utilizes phosphorylation as a reversible mechanism to assemble and dissociate the tegument at different stages of viral replication (35, 36).

The acidic cluster of UL11 (Fig. 1) contains the major site of phosphorylation (24), and it is possible that use of this serine might be the controlling residue. Alternatively, it is possible that the phosphorylation states of UL11 tegument protein are gE from varicella-zoster virus (28, 33) and gB from human cytomegalovirus, for which an acidic cluster motif is required (44, 55). Further studies of the trafficking pathways used by herpesvirus proteins are needed to provide mechanistic explanations for these differences.

**ACKNOWLEDGMENTS**

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