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Biochemical, Biophysical, and Mutational Analyses of Subunit Interactions of the Human Cytomegalovirus Nuclear Egress Complex

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Received 26 November 2008/Accepted 8 January 2009

Nuclear egress, the trafficking of herpesvirus nucleocapsids from the nucleus to the cytoplasm, involves two conserved viral proteins that form a complex at the nuclear envelope, referred to as the nuclear egress complex. In human cytomegalovirus, these two proteins are called UL50 and UL53. To study UL50 and UL53 in molecular detail, these proteins were expressed in bacteria and purified. To obtain highly expressed, pure proteins, it was necessary to truncate both constructs based on sequence conservation and predicted secondary structural elements. Size exclusion chromatography and analytical ultracentrifugation studies indicated that the truncated form of UL50 is a monomer in solution, that the truncated form of UL53 is a homodimer, and that, when mixed, the two proteins form a heterodimer. To identify residues of UL53 crucial for homodimerization and for heterodimerization with UL50, we constructed and expressed mutant forms of UL53 containing alanine substitutions in a predicted helix. Isothermal titration calorimetry was used to measure the binding affinities of the UL53 mutants to UL50. UL53 residues, the replacement of which reduced binding to UL50, form a surface on one face of the predicted helix. Moreover, most of the substitutions that reduce UL53-UL50 interactions also reduced homodimerization. Substitutions that reduced the interaction between UL50 and UL53 in vitro also reduced colocalization of full-length UL50 and UL53 at the nuclear rim in transfected cells. These results demonstrate direct protein-protein interactions between these proteins that are likely to be mediated by a helix, and they have implications for understanding nuclear egress and for drug discovery.

The delivery of newly assembled herpes capsids from the nucleus to the cytoplasm is a known process known as nuclear egress (12). This process is rather poorly understood. Although it is generally recognized that early steps of nuclear egress entail nucleocapsids gaining access to the inner nuclear membrane, followed by primary envelopment of the nucleocapsids and budding through the inner nuclear membrane, there are currently three models for the remaining steps (13, 14). Even the early steps in nuclear egress are not understood mechanistically although the formation of a specific nuclear egress complex (NEC) (10, 11, 19, 21) has been shown to be crucial. In alphaherpesviruses such as herpes simplex virus (HSV) and pseudorabies virus (PRV), the NEC consists of two proteins called UL34 and UL31 (11). Throughout the alpha, beta, and gamma subfamilies of herpesviruses, highly conserved homologs of these two proteins are found to form an NEC at the nuclear rim in all studied cases (1, 4, 5, 8, 10, 17–19, 25). Remarkably, in transfected cells, the PRV NEC, without any nuclear rim in all studied cases (1, 4, 5, 8, 10, 17–19, 25). These studies, however, did not exclude the formal possibility that NEC interactions are mediated by cellular components, they did not ascertain the quaternary structure of the complex, and they did not measure the thermodynamics and affinities of the interactions. Here, we study the UL50-UL53 complex using a combination of biochemical, biophysical, and cytochemical techniques to compare the properties of wild-type (wt) and mutant proteins and identify specific interactions that mediate formation of the HCMV NEC.

MATERIALS AND METHODS

Plasmids. The open reading frames coding for UL50 residues 1 to 349 (lacking the C-terminal 48 residues, which include the transmembrane region [UL50Δ349]) and UL53 residues 1 to 376 (full-length UL53) from HCMV strain AD169 were amplified by PCR from AD169 DNA with Platinum Pfx DNA Polymerase from Invitrogen (for primers used in this study, see Table 1 posted at http://coen.med.harvard.edu/supplementaldata.htm) and cloned into an IMPACT-CN pTYB12 expression vector that utilizes the intein protein from *Saccharomyces cerevisiae* (New England Biolabs) linked to a chitin binding domain as an inducible self-cleavage fusion tag. Here, the N terminus of each target protein was fused to the C terminus of the intein using NdeI and EcoRI sites. Upon cleavage of the fusion protein, this system produces a target protein with...
three additional amino acids (AlaGlYHis) upstream of the N-terminal methionine residue. Subsequently, truncations of UL50 (UL50Δ208 and UL50Δ169 consisting of residues 1 to 208 and 1 to 169, respectively) were engineered from the original constructs by introducing internal stop codons using QuikChange (Stratagene), whereas truncations of UL53 (the constructs UL53Δ221, UL53Δ292, and UL53Δ50, consisting of residues 1 to 231, 1 to 292, and 30 to 292, respectively) were engineered by PCR amplification, and cloned into a pGEX-6P-1 vector using the BamHI and EcoRI restriction sites. The genes coding for full-length hemagglutinin (HA)-UL50 and FLAG-UL53 were amplified by PCR from AD169 DNA using primers encoding HA and FLAG tags, respectively, at the 5′ end of forward primers (see Table 1 posted at http://coen.med.harvard.edu/supplementaldata.htm) with KOD DNA Polymerase (Novagen) and cloned into a pECDNA3 vector using the KpnI and EcoRI restriction sites. A start codon was introduced into the pECDNA vector immediately upstream of the KpnI site, replacing the HindIII sequences.

Engineered plasmids were sequenced using the Dana Farber Cancer Institute, Molecular Biology Core Facility, to confirm the existence of the engineered changes and no others.

**Protein expression and purification.** Plasmids encoding full-length UL53, carboxy-terminal truncations of UL50 and UL53, and variants of UL53 with alanine substitutions were transformed into BL21(DE3) CodonPlusRP (Stratagene) and grown in 4 to 6 liters of Luria-Bertani medium, and cells were induced for overexpression by the addition of 0.3 mM isopropyl-thiogalactopyranoside (IPTG) for 16 to 24 h at 30°C. Protein expression and purification were carried out in a total of 12 s with a 210-s delay between injections. Each protein sample (UL53Δ169, UL53Δ50, UL53Δ79A, UL53Δ84, and UL53Δ169-UL53Δ50 complex) was examined at different concentrations ranging from 70 to 120 μM. The titrations were stopped when the baseline was reached. The temperature of the calorimeter was controlled to ±0.1°C. Each heat pulse was recorded, and the integrals of these heat pulses were plotted for the UL50Δ169-UL53Δ50 complex, in a similar manner. Whenever a complex formed under these conditions, the apparent molecular mass and Stokes radius of the complex could be estimated. In cases where complex formation was observed, the dissociation constant (K_D) of the complex could be estimated to be less than the concentration of the complex that was loaded onto the column.

**Quantitative binding affinities were measured using a VP-ITC calorimeter (MicroCal Inc.).** Protein samples used in ITC experiments were prepared in a buffer containing 20 mM NaCl, 25 mM HEPES, pH 7.0, and 1 mM DTT (buffer C). For each AU experiment, 120 μl of protein was loaded into a 3- by 3-cm cuvette, and an additional 120 μl of buffer C was loaded alongside in a blank cuvette for background subtraction. The output data represent the distributions of the protein within the solution column measured at 280 nm when the sample reached equilibrium at a specific centrifuge speed. The distribution of the molecules along the solution column can be described as an exponential function of the buoyant mass of the molecule of interest, by the following equation: 

\[ A_0 = A_{oph}(M - \rho_0)/(\rho - \rho_0) \]

where \( A_0 \) is the absorbance of solutes at any radial distance, \( A_{oph} \) is the absorbance of the solute at the reference radial distance \( \rho_0 \) is the rotational velocity in radians/s, \( r \) is the universal gas constant, \( T \) is the absolute temperature, \( M \) is the molecular mass, \( \rho \) is the density of the solution, \( \rho_0 \) is the partial specific volume of the solute, and \( \rho \) is the density of the solution. The values for \( \rho_0 \) and \( \rho \) were determined to be 0.73 and 1.0076, respectively. All data were fit to the above equation using multiple fit alignment from the XL-I software package (Beckman Coulter), which assumed that the sample contains a single species.

**NMR spectroscopy.** One-dimensional (1D)-{1H}-nuclear magnetic resonance (NMR) spectra for wt UL53Δ50, UL53Δ50-L61A, UL53Δ50-L74A, UL53Δ50-L79A, UL53Δ50-M82A, and UL53Δ50-E73A/K77A were acquired at 285.3 K on a Bruker DRX 600 MHz spectrometer equipped with a xyz-gradient triple-resonance probe. Protein sample concentrations were ~150 to 300 μM in 25 mM HEPES, pH 7.0, 200 mM NaCl, 1 mM DTT, and 5% D_2O.

**ITC.** Quantitative binding affinities were measured using a VP-ITC calorimeter (MicroCal Inc.). Protein samples used in ITC experiments were prepared in buffer B. In each binding experiment, UL50Δ169 was placed in the syringe (at concentrations ranging from 70 to 120 μM), and UL53Δ50 was placed in the reservoir (at concentrations ranging from 1.2 to 12 μM). The titrations were performed at 25°C with a mixing speed of 270 rpm, and each injection was carried out in a total of 12 s with a 210-s delay between injections. Each ITC experiment consisted of 30 injections at 10 μl per injection after an initial injection of only 1.5 μl that was excluded from the fit. Each injection gave rise to an exothermic heat pulse; the integrals of these heat pulses were plotted for the series of all 30 injections against the molar ratio of UL50Δ169 to UL53Δ50, thereby producing the binding curve. The resulting isotherms were fit to a
RESULTS

Expression of UL50 and UL53. UL53 and in particular UL50 polypeptides are poorly soluble in their full-length (8) and near-full-length forms, respectively (3). Initial attempts to express and purify the full-length UL53 and UL50Δ310 constructs as maltose binding protein (MBP) fusion proteins were unsuccessful, mainly because multiple truncated species were produced. In addition, it was difficult to remove the MBP completely without causing the target protein to precipitate out of solution. For these reasons, we decided to try removing sequences lying outside of secondary structural elements and regions of the proteins that are conserved among herpesviruses (predicted using the Protein Homology/analogY Recognition Engine, PHyre, at www.sbg.bio.ic.uk/~phyre/).

For UL53, three truncations were made (Fig. 1A) to yield a final polypeptide that gave robust expression with the expected molecular mass as judged by SDS-PAGE (Fig. 1C, lane 7). Similarly, four different truncations were tried for UL50 before a polypeptide that could be successfully expressed and purified (Fig. 1B, and C, lane 6) was found. The final constructs for UL50 and UL53 were UL50Δ169 (Fig. 1B) and UL53Δ50 (Fig. 1A), respectively. Other than the transmembrane segment of UL50 and nuclear localization signal of UL53, the constructs contain all segments that are conserved among the various herpesvirus NEC proteins. Both UL50Δ169 and UL53Δ50 were expressed and purified as chitin binding domain-intein fusion proteins because this inducible self-cleavage system permits the isolation of the target proteins with a single affinity column without the use of an added protease. Other fusion tags (glutathione-S-transferase, MBP, and His6) were explored with little success due to nonspecific aggregation either with the tagged proteins or with endogenous proteins during purification (data not shown).

UL50Δ169 heterodimerizes with UL53Δ50. The apparent molecular masses and Laurent and Killander Stokes radii of UL50Δ169, UL53Δ50, and UL50Δ169-UL53Δ50 complex (Table 1) were determined using size exclusion chromatography, based on standards run on the same column (Fig. 2A and B). The calculated molecular masses of UL50Δ169 and UL53Δ50, based on their primary sequences, are 19.3 kDa and 28.0 kDa, respectively. UL50Δ169 fractionated between the position of cytochrome c (12.4 kDa) and carbonic anhydrase (29 kDa)
with an apparent molecular mass of ~17 kDa, which is consistent with UL50Δ169 being a monomer in solution (Fig. 2A). UL53Δ50 fractionated between carbonic anhydrase and albumin (66 kDa) with an apparent molecular mass of ~42 kDa, in between the expected mass for a monomer and a homodimer (Fig. 2A) (see below for further analysis). When UL50Δ169 and UL53Δ50 were mixed at a 1:1 molar ratio and loaded onto the same size exclusion column, a single symmetrical peak was observed (data not shown) with an apparent molecular mass of ~37 kDa (Fig. 2A). SDS-PAGE analysis of fractions from this peak showed that it contained both proteins (Fig. 2C). Given that the apparent molecular mass of this complex was smaller than the sum of the masses of each component (47 kDa), the results are most consistent with the explanation that UL50Δ169 and UL53Δ50 form a heterodimer rather than a higher-order complex. The K_d for formation of this complex could be estimated as being lower than the concentration (1.9 μM) of the complex loaded onto the column (Table 2).

**AU analysis of homo- and heterodimers.** As an alternative test of complex formation, we analyzed UL53Δ50 and the UL50Δ169-UL53Δ50 complex using sedimentation equilibrium in an analytical ultracentrifuge. To ensure that data acquired using this technique could be correlated to those acquired using size exclusion chromatography, AU samples were prepared from peak fractions eluted from the Superdex 200 column. This technique offers advantages over size exclusion chromatography including the following: (i) measurements are performed on protein that is free in solution, which eliminates possible protein interactions with the matrices and surfaces of the gel filtration matrix; and (ii) the shape of the protein has no effect on estimating its mass because the mass is estimated from the distribution of the protein along the solution column at equilibrium when the rate of sedimentation is balanced by the rate of diffusion. The molecular masses of UL53Δ50 and the UL50Δ169-UL53Δ50 complex were determined by collecting data at multiple protein concentrations and centrifuge speeds (Fig. 3A and B and Table 1). Specifically, the apparent molecular masses of UL53Δ50 and UL50Δ169-UL53Δ50 complex were estimated from nine and seven independent sedimentation equilibrium experiments, respectively. In both cases, the data obtained were fit to exponential functions for an ideal noninteracting single component (see equation in Materials and Methods). When wt UL53Δ50 was mixed with wt UL50Δ169 in a 1:1 molar ratio, a single symmetrical peak was observed on the Superdex 200 column (data not shown). Fractions from this peak showed coelution of the two proteins, as judged on an SDS-polyacrylamide gel (C), whereas UL53 lacking residues 1 to 83 (UL53Δ84) (D) or a UL53Δ50-L79A mutant protein (E) did not coelute with UL50Δ169 when mixed at similar concentrations as the wt proteins. The positions of the various proteins are indicated to the right of the images of the gels.

**TABLE 1.** Protein molecular masses estimated using AU sedimentation equilibrium and size exclusion chromatography

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass by method of determination (kDa)</th>
<th>Stokes radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary sequence</td>
<td>Size exclusion</td>
</tr>
<tr>
<td>UL53Δ50</td>
<td>28.0</td>
<td>42</td>
</tr>
<tr>
<td>UL50Δ169-UL53Δ50</td>
<td>47.3</td>
<td>37</td>
</tr>
<tr>
<td>UL53Δ84</td>
<td>28.0</td>
<td>37</td>
</tr>
<tr>
<td>UL50Δ169</td>
<td>23.8</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>19.3</td>
<td>17</td>
</tr>
</tbody>
</table>

*a* Calculated molecular mass based on primary sequence.

*b* Apparent molecular mass determined from a Superdex 200 column.

*c* As determined from data obtained from a Superdex 200 column.

*d* ND, not determined.

**FIG. 2.** Size exclusion chromatography analysis of UL50, UL53, and the UL50-UL53 complex. (A) The logs of the molecular masses (MM) of protein standards were plotted as a function of their elution volumes from the Superdex 200 column and fit to a straight line. The positions of the protein standards in this plot are indicated by diamond symbols surrounded by circles. The molecular masses of the standards, starting from the left, are 200 kDa (β-amylase), 150 kDa (alcohol dehydrogenase), 66 kDa (albumin), 29 kDa (carbonic anhydrase), and 12.4 kDa (cytochrome c). Diamonds corresponding to UL50Δ169, UL53Δ50, UL53Δ50-50Δ169 complex (53Δ50-50Δ169), UL53Δ50-L79A (53Δ50-L79A), and UL53Δ84 (53Δ84, where residues 1 to 83 are missing) were placed on the standard line according to their elution volumes, in order to measure their apparent molecular masses. (B) The Stokes radii of the protein standards were plotted against their (−logKav)½ values to generate a standard plot. The Stokes radii of 50Δ169, 53Δ50, 53Δ50-50Δ169 complex, 53Δ50-L79A, and 53Δ84 were also determined from this plot based on their calculated (−logKav)½ values (see Materials and Methods). When wt UL53Δ50 was mixed with wt UL50Δ169 in a 1:1 molar ratio, a single symmetrical peak was observed on the Superdex 200 column (data not shown). Fractions from this peak showed coelution of the two proteins, as judged on an SDS-polyacrylamide gel (C), whereas UL53 lacking residues 1 to 83 (UL53Δ84) (D) or a UL53Δ50-L79A mutant protein (E) did not coelute with UL50Δ169 when mixed at similar concentrations as the wt proteins. The positions of the various proteins are indicated to the right of the images of the gels.
yielded an average molecular mass for UL53 and Methods). A typical plot for each protein sample is provided in Fig. 3A and B. Collectively fitting all of these data yielded an average molecular mass for UL53Δ50 of 54 kDa and 46 kDa for the UL50Δ169-UL53Δ50 complex (Table 1). These molecular mass estimates based on analytical centrifugation agree closely with the calculated molecular masses of the homodimer (56.0 kDa) and heterodimer (47.3 kDa). Taking the size exclusion chromatography and AU results together, we conclude that UL53Δ50 forms a homodimer in the absence of UL50Δ169 and a UL50Δ169-UL53Δ50 heterodimer when UL50Δ169 is present.

**Residues 50 to 83 of UL53 are necessary for homodimerization.** In the process of determining whether UL53 includes protease-resistant domain(s), we treated UL53Δ50 with trypsin. A large contiguous fragment of UL53 was identified that was resistant to trypsin digestion. Mass spectroscopy and N-terminal sequencing showed that this fragment had a molecular mass estimates based on analytical centrifugation of 46 kDa for the UL50Δ50 complex (Table 1). These molecular mass estimates based on analytical centrifugation agree closely with the calculated molecular masses of the homodimer (56.0 kDa) and heterodimer (47.3 kDa). Taking the size exclusion chromatography and AU results together, we conclude that UL53Δ50 forms a homodimer in the absence of UL50Δ169 and a UL50Δ169-UL53Δ50 heterodimer when UL50Δ169 is present.

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<table>
<thead>
<tr>
<th>UL53 protein</th>
<th>Complex formation by SEC</th>
<th>Estimated column concn (μM)</th>
<th>Kd (μM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>Yes</td>
<td>1.9</td>
<td>0.29 ± 0.03</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>D63A/D66A/E70A</td>
<td>Yes</td>
<td>9.0</td>
<td>0.16 ± 0.01</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>I67A</td>
<td>ND‡</td>
<td>ND</td>
<td>0.32 ± 0.07</td>
<td>0.62 ± 0.0</td>
</tr>
<tr>
<td>H62A</td>
<td>ND</td>
<td>ND</td>
<td>0.52 ± 0.19</td>
<td>0.83 ± 0.0</td>
</tr>
<tr>
<td>L76A</td>
<td>ND</td>
<td>ND</td>
<td>0.60 ± 0.00</td>
<td>0.83 ± 0.0</td>
</tr>
<tr>
<td>L74A</td>
<td>Yes</td>
<td>4.0</td>
<td>0.63 ± 0.02</td>
<td>0.80 ± 0.0</td>
</tr>
<tr>
<td>R69A</td>
<td>ND</td>
<td>ND</td>
<td>0.98 ± 0.01</td>
<td>0.83 ± 0.0</td>
</tr>
<tr>
<td>E73A/K77A</td>
<td>Yes</td>
<td>3.5</td>
<td>1.2 ± 0.04</td>
<td>1.04 ± 0.0</td>
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<tr>
<td>L61A</td>
<td>Yes</td>
<td>5.1</td>
<td>1.7 ± 0.07</td>
<td>0.95 ± 0.2</td>
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<tr>
<td>H71A</td>
<td>ND</td>
<td>ND</td>
<td>2.4 ± 0.51</td>
<td>0.93 ± 0.1</td>
</tr>
<tr>
<td>L64A</td>
<td>ND</td>
<td>ND</td>
<td>4.1 ± 0.80</td>
<td>0.83 ± 0.0</td>
</tr>
<tr>
<td>F68A</td>
<td>ND</td>
<td>ND</td>
<td>9.5 ± 0.00</td>
<td>0.66 ± 0.0</td>
</tr>
<tr>
<td>M82A</td>
<td>Partial</td>
<td>4.3</td>
<td>−50</td>
<td>ND</td>
</tr>
<tr>
<td>E75A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L79A</td>
<td>No</td>
<td>4.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

— SEC, size exclusion chromatography.
— Kd, dissociation constant.
— N, binding stoichiometry.
— ND, not determined.

Although the low apparent molecular masses of L79A, L64A, I67A, and F68A could be due to major alterations in the shape of the complexes, a more likely explanation is that dimers of the mutant proteins are in a rapidly exchanging equilibrium with monomers.

We therefore used AU equilibrium sedimentation to probe the oligomerization state of UL53Δ50-L79A. The AU data for UL53Δ50-L79A were acquired at concentrations similar to those used for the wt UL53Δ50 protein. The AU data fit best to parameters for an equilibrium between a UL53Δ50 monomer (28 kDa) and a dimer (56 kDa) (Fig. 3C), with a monomer/dimer ratio of about 1:3. When we attempted to fit these data to a model for an ideal single species, the apparent molecular mass was ~42 kDa (data not shown), which lies between the calculated molecular masses of the monomer and dimer. Taken together, the data indicate that the L79 side chain and probably L64, I67, and F68 are necessary for formation of the UL53Δ50 homodimer in solution.

**Specific amino acids of UL53 required for interaction with UL50.** Lötzerich et al. (10) have shown that MCMV M53 mutations that affect a segment corresponding to residues 61 to 82 of UL53 decrease coimmunoprecipitation and colocalization of M50 with M35. We therefore tested the UL38A48 polypeptide for its ability to interact with the wt UL50Δ169 protein. UL53Δ84 and UL50Δ169 were mixed at a 1:1 molar ratio and were fractionated using a size exclusion column; the concentration of each protein in the column was approximately 10 μM. At these concentrations, UL53Δ84 failed to coelute with UL50Δ169 (Fig. 2D). Furthermore, the UL53Δ50-L79A mutant also failed to interact with UL50Δ169 during size exclusion chromatography at similar concentrations (Fig. 2E). To investigate whether the L79A substitution interferes with correct folding of the protein, a 1D-1H-NMR spectrum was acquired and compared to the spectrum of the wt protein. The spectra were very similar and exhibited proton chemical shifts that are indicative of folded proteins, including the peaks arising from methyl protons between −1 and 1 ppm and the wide dispersion of amide proton chemical shifts between 6 and 10 ppm (Fig. 5A and F). The small differences observed in the spectra may reflect conformational changes in UL53 during its transition between its monomeric and dimeric forms at equilibrium. Regardless, these results taken together suggest that residues 50 to 84 of UL53 are required to interact with UL50 and that the L79 side chain plays a critical part in that interaction.

Secondary structure prediction using PHyre suggested that residues 61 to 82 of UL53 have a propensity to form a long alpha helix. The presence of a proline residue in the middle of this sequence may introduce flexibility to the helix (Fig. 6A). To test whether the interaction of UL53 with UL50 might involve one face of this predicted helix, we replaced most of the side chains of this segment with alanines, one at a time. Additionally, a triple substitution was engineered in residues predicted to lie on the face of the helix opposite to the L79 side chain (Fig. 6A). Based on the hypothesis that side chains lying on the surface opposite to that of L79 would not play a role in mediating interaction with UL50Δ169. All engineered mutants were purified in a manner similar to that of the wt protein. 1D-1H-NMR spectra were collected for selected mutants and compared to the spectrum of the wt protein (Fig. 5). Again, the...
proton chemical shifts of the wt and mutant proteins showed remarkable similarity. In addition, a subset of the engineered UL53Δ50 mutants (L64A, F68A, H71A, and E75A) exhibited symmetrical peaks on size exclusion chromatography, and two of these mutants (H71A and E75A) were not impaired for homodimerization (Fig. 4).

As an initial test of whether the mutant proteins could heterodimerize with UL50Δ169, some of the UL53Δ50 mutant

FIG. 3. AU analysis. Experimental data (open circles) from representative AU analyses of 0.93 mg/ml UL53Δ50 (A), 0.95 mg/ml UL50Δ169-UL53Δ50 complex (B), and 0.73 mg/ml UL53Δ53-L79A point mutant (C) are presented. The data were fit to an exponential function described in Materials and Methods, and the residual errors of the fitted data are shown above each plot.

FIG. 4. Effects of mutations on homodimerization of UL53Δ50. (A) Chromatographic profiles of wt UL53Δ50 (WT), UL53Δ50-L64A (L64A), UL53Δ50-I67A (I67A), and UL53Δ50-F68A (F68A) from a Superdex 75 size exclusion column. (B) Chromatographic profiles of wt UL53Δ50 (WT), UL53Δ50-H71A (H71A), and UL53Δ50-E75A (E75A) from a Superdex 75 size exclusion column. In both panels, the positions of the arrows correspond to the elution volumes of the protein standards (molecular masses indicated) used to calibrate the column. Abs, absorbance.
proteins were mixed with UL50/H9004169 and chromatographed on Superdex 200. Of the UL53/H900450 mutants tested, UL53/H900450-L79A and, to lesser extent, UL53/H900450-M82A were incapable of forming a complex with UL50/H9004169 at the concentrations listed in Table 2. Thus, qualitatively, these two mutants were impaired for heterodimerization.

To investigate this issue more quantitatively, the binding of wt UL53/H900450 and the UL53/H900450 mutants to wt UL50/H9004169 was measured using ITC. A typical example of such a titration is shown in Fig. 6B for the wt proteins, which reveals an exothermic binding reaction. Fitting these data gave a dissociation constant ($K_d$) for the complex of 0.29 ± 0.03 µM (Fig. 5B and 6B).
Table 2). For the wt proteins, the interaction had a $\Delta G$ of $-8.9$ kcal/mol, a $\Delta H$ of $-13$ kcal/mol, a $-T\Delta S$ of $4.1$ kcal/mol, and a reaction stoichiometry (N) of 1.0 ± 0.1. The $K_f$ and $N$ values for each UL53 mutant tested are reported in Table 2. Additionally, the titration of the UL53Δ50-L79A mutant to UL50Δ169 was indistinguishable from that of a negative control where UL50Δ169 was replaced with buffer (Fig. 6C). The affinities of UL53Δ50 mutants for wt UL50Δ169 fell into three categories: (i) those that are completely defective or retain little binding (with $K_f$ values >30-fold higher than those of the wt and mutants E75A, L79A, M82A, and F68A), (ii) those that are moderately defective (with $K_f$ values 5- to 10-fold higher than those of the wt and mutants L64A, H71A, L61A, L74A, and E73A/K77A), and (iii) those that retain wt or near wt binding affinity (with $K_f$ values less than four fold higher than those of the wt and mutants H62A, R69A, L76A, I67A, and D63A/D66A/E70A) (Table 2). The ITC data were in complete agreement with the more qualitative data acquired using Superdex 200 size exclusion columns (Table 2). The residues whose substitution reduced heterodimerization all lie on one face of the predicted alphahelix shown in Fig. 6A.

UL53 substitutions that diminish heterodimerization with UL50 also affect colocalization of the NEC in transfected cells. When UL50 is expressed in transfected cells, it is predominantly localized to the nuclear rim, whereas UL53 expressed in the absence of UL50 is located diffusely within the nucleus (2, 15). When both proteins are coexpressed, UL53 colocalizes with UL50 at the nuclear membrane (2, 15). To test whether mutations that substantially decreased heterodimerization in the in vitro ITC assay affected NEC formation in transfected cells, wt, M82A, and L79A versions of UL53 were engineered into a pcDNA3 vector that expresses the full-length UL50 with an N-terminal HA epitope tag. M82A reduced heterodimerization affinity 170-fold, and L79A led to a reduction of >170-fold (Table 2). A plasmid expressing full-length wt UL50 with an N-terminal HA epitope tag was also constructed. Constructs expressing wt or mutant forms of UL53 and wt UL50 were transfected into 293 cells, either alone or together for coexpression, and the cellular localization of the proteins was assessed by immunofluorescence 48 h post-transfection (Fig. 7). Individualy expressed wt UL50 was detected in the nucleus with stronger staining of the nuclear rim, and there was also perinuclear staining (Fig. 7A). Individually expressed UL53 predominantly exhibited a diffuse intranuclear localization (Fig. 7B). When the proteins were coexpressed, the localization of UL53 coincided with that of UL50 at the nuclear rim, and intranuclear and perinuclear staining of UL50 appeared to be diminished (Fig. 7C). These results and, in particular, the strong influence of UL50 on the nuclear localization of UL53 are consistent with previous observations by Milbradt et al. and Camozzi et al. (2, 15). When the singly substituted UL53 constructs were cotransfected with wt UL50, little if any of L79A partitioned to the nuclear rim despite the presence of UL50 (Fig. 7D), and only partial distribution of UL50 to the nuclear rim was observed, similar to the staining of singly transfected UL50. M82A partially partitioned to the nuclear rim with UL50, but a substantial fraction retained a diffuse nuclear localization (Fig. 7E). Thus, in the context of full-length UL53, substitutions that decreased binding to UL50 in vitro also decreased colocalization of UL53 with UL50 at the nuclear rim in cells.

**DISCUSSION**

Nuclear egress is a crucial stage of the replication cycle of herpesviruses and, in turn, the NEC is a crucial component in this process. Formation of the HCMV NEC requires two key proteins, UL50 and UL53. To analyze these proteins biochemically and biophysically, we found it necessary to remove sequences outside of regions that are conserved among herpesviruses. These nonconserved sequences that were removed are predicted to adopt random-coil secondary structures. We have therefore expressed, purified, and characterized truncated forms of the UL50 (UL50Δ169) and UL53 (UL53Δ50) proteins, which retain all segments conserved among herpesviruses, and studied their molecular interactions in vitro.

**Formation of the HCMV NEC is mediated via direct protein-protein interactions.** Previous studies of herpesvirus NECs have strongly suggested that the two virally encoded components interact directly. This suggestion was derived from the ability to coimmunoprecipitate one component with an antibody directed against the other in transfected cells (1, 4, 5, 8, 10, 17, 20). In addition, interactions between HCMV UL50 and UL53 have been detected in a yeast two-hybrid assay (15). However, these studies have not excluded the formal possibility that interactions between NEC components could require cellular proteins that are highly conserved between yeast and mammals. Our results, using highly purified UL50 and UL53 expressed in bacteria, overcome this formal possibility and demonstrate a direct interaction between these two NEC components. Furthermore, our finding that substitutions that reduce or abolish the interaction in vitro also inhibit colocalization of the two proteins at the nuclear rim in transfected cells supports the concept that this direct interaction mediates NEC formation in cells.

**HCMV UL50 and UL53 form a heterodimer.** To our knowledge, the quaternary structure of any herpesvirus NEC has not been examined previously. In this study, we have shown that while UL50Δ169 is a monomer in solution and UL53Δ50 is a homodimer, the two proteins form a heterodimer when they are mixed at a 1:1 molar ratio. Our results further suggest that the affinity of UL50Δ169 for UL53Δ50 is stronger than the interaction of the UL53Δ50 homodimer. Indeed, the $K_f$ for heterodimerization was in the high-nanomolar range compared to an estimated $K_f$ for homodimerization in the low-micromolar range (data not shown). The results also suggest that UL50Δ169 competes for a surface on UL53Δ50 that overlaps the surface required for mediating the UL53Δ50 homodimerization. This suggestion is supported by the effects of single UL53 substitutions that block both homodimerization and heterodimerization with UL50.

The apparent molecular masses and Stokes radii of all proteins tested (UL50Δ169, UL53Δ50, UL53Δ50-L79A, UL53Δ84, and UL50Δ169-UL53Δ50 complex) derived from size exclusion chromatography data were smaller than the calculated molecular masses and Stokes radii based on the sequence and association states of the proteins (Table 1). The differences between the apparent and calculated masses were greatest for the UL53Δ50 homodimer while only a modest
difference was seen with the UL50Δ169 monomer. Because estimates of molecular masses using size exclusion chromatography are based on the assumption of spherical shape, the finding of low apparent molecular weight may suggest that the proteins deviate significantly from spherical shape. However, because deviations from spherical shape most often result in more rapid migration in size exclusion chromatography, a more likely explanation is that the proteins are retarded due to interactions with the resin.

Our finding that UL50Δ169 and UL53Δ50 form a heterodimer suggests, in turn, that full-length UL50 and UL53 may function as a heterodimer in the NEC in infected cells. We caution that we cannot rule out the possibility that segments of UL50 and UL53 that have been removed from our truncated constructs mediate higher-order structures. Nevertheless, the truncated proteins contain all of the segments that are conserved among various herpesvirus NECs, suggesting that the constructs retain the important structural and functional features of the NEC.

**UL53 heterodimerization with UL50 involves one face of a predicted alpha-helix.** Our results utilizing trypsin digestion and mutational analysis identified a segment within UL53Δ50 that is required for interaction with UL50Δ169. Within this segment, residues 60 to 82 are predicted to form an alpha-helix. Our finding that alanine substitutions that decrease interaction with UL50Δ169 all lie on one face of the predicted helix provides evidence that this segment is indeed alpha-helical. We hypothesize then that this helix exists and that it binds directly to UL50. The residues on this predicted helical face that are important for heterodimerization include mostly hydrophobic residues (L61, L64, F68, L79, and M82) and two charged residues (E75 and H71A). This suggests that the in-
teration between UL50 and UL53 is mediated by a combination of hydrophobic and charge-charge interactions.

It is interesting to compare our results regarding heterodimerization with the thorough analysis of Lotzerich et al. (10) of the effects of substitutions in a segment of MCMV M53 that is homologous to residues 61 to 82 of HCMV UL53. In keeping with our results, the I133A M53 mutant had a substantial effect on coimmunoprecipitation and colocalization with M50, much as the corresponding M82A UL53 substitution had a major effect on heterodimerization with UL50 in vitro and on colocalization with UL50 in transfected cells. The M53-L130A substitution, which corresponds to the UL53-L79A substitution, greatly reduced colocalization of NEC components at the nuclear rim, which is similar to our results. However, this M53 substitution did not prevent coimmunoprecipitation of the two components following expression in transfected cells, while the UL53-L79A substitution had the most drastic effect of all the mutations on heterodimerization in vitro, increasing the $K_d$ value $>170$-fold. The individual M53 substitution that exerted the greatest effect was Y129A. We found that the equivalent UL53 substitution (Y78A) could not be studied in vitro due to insolubility, raising the possibility that poor protein folding could have influenced the phenotype of the MCMV Y129A mutant in cells. On the other hand, a number of equivalent substitutions showed rather different phenotypes. For example, we found that the UL53-E75A substitution had a drastic effect on heterodimerization while the equivalent M53-E126A substitution exerted no effect in colocalization or coimmunoprecipitation assays. Thus, although the homologous segments of M53 and UL53 both seem to be important for subunit interactions within the NEC, equivalent residues appear to have different degrees of importance for each interaction. Indeed, M50 is not capable of forming a complex with UL53 (data not shown), even though these two proteins share homology within the regions that interact with their respective partner proteins to form the NECs. This may be relevant to the finding that although UL50 and UL53 homologues have similar functions within each herpesvirus, the homolog from any one subfamily cannot complement the defects of a null mutant from another subfamily (21).

The UL53 homodimer. Our data, particularly the AU analysis, show that UL53 forms a homodimer. This finding stands in contrast to the failure of Milbradt et al. to observe UL53-UL53 interactions in a yeast two-hybrid assay (15). However, it is not uncommon for proteins known to interact in a physiologically relevant manner to score negative in that assay. Of the residues that we tested that are important for UL50-UL53 heterodimerization (L64, F68, and L79), all were also important for UL53 homodimerization. Thus, heterodimerization and homodimerization appear to be mediated by the same surface of an alpha-helix. Nevertheless, despite the above-mentioned conservation of the predicted helix between HCMV and MCMV, we did not observe homodimerization of M53 in vitro (unpublished results). It is interesting that I67A, which lies on the edge of the heterodimerization surface, is the only substitution that we found to have an effect on UL53-UL50 homodimerization while retaining wild-type or nearly wild-type binding to UL50Δ169.

The EBV BFLF2 homolog of UL53 is involved in primary envelopment of nuclear capsids and has also been reported to be required for DNA cleavage/packaging (6). Based on the multiple functions attributed to EBV BFLF2, it is tempting to speculate that UL50 and UL53 may have additional functions other than primary envelopment at the inner nuclear membrane and, in particular, that the homodimer of UL53 may play a particular biological role. However, it is possible that the UL53 homodimer has evolved simply for solubility and stability when UL50 is not yet present in sufficient amounts during infection to form the heterodimer that is presumed to be the functional unit of the NEC. In this context, it may be relevant that the amino acids of UL53 that we have shown to interact with UL50 include several hydrophobic residues. These amino acid side chains might produce a hydrophobic patch on the protein surface that would be unfavorable to expose to the aqueous solvent and that might tend to stick to membranes nonspecifically.

Potential implications. HCMV is an important pathogen, particularly in the immunocompromised and neonatal populations (16). Although there are a number of anti-HCMV drugs available, all have drawbacks in terms of toxicity and/or bioavailability (23). Nuclear egress is a process that is not known to mimic any cellular process, and the UL50 and UL53 families of proteins are unique to herpesviruses and conserved within each family. Work in the MCMV system indicates that single M50 substitutions (1) and clustered M53 substitutions (10) that interrupt NEC subunit interactions are lethal. Our results indicate that a single amino acid substitution can drastically reduce UL50-UL53 heterodimerization. This suggests that a small molecule could also disrupt this interaction and thus exert antiviral activity. Such compounds could have the potential to be both highly selective and potent.

Nuclear egress is a very poorly understood process. We know very little regarding how the NEC promotes primary envelopment at the inner nuclear membrane. The observation that the PRV NEC, without any other viral proteins, can induce the formation of vesicles akin to the products of primary envelopment (7) suggests that the NEC can carry out complicated interactions between proteins and membranes. Our findings should serve as a good starting point for furthering our understanding of nuclear egress on a biophysical and biochemical basis.

ACKNOWLEDGMENTS

We thank Jawdat Al-Bassam for his generous help with collecting and analyzing the AU data, Chikako Suzuki and Hari Arthanari for their help in acquiring the NMR spectra, Sofia Hamirally for early work on cloning and expressing UL50 and UL53, Igor Jurak for his help with preparing samples for immunofluorescence microscopy, and the Nikon Imaging Center at Harvard Medical School for training and the use of the 80i microscope.

B.T.E. was supported by the Summer Honors Undergraduate Research Program. This work was supported by the National Institutes of Health grant R01 AI1026077 to D.M.C. and J.M.H.

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