SPECIFIC RESIDUES OF A CONSERVED DOMAIN IN THE N-TERMINUS OF THE HUMAN CYTOMEGALOVIRUS pUL50 PROTEIN DETERMINE ITS INTRANUCLEAR INTERACTION WITH pUL53

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Capsule

Background: Interaction between the cytomegalovirus proteins pUL50 and pUL53 is essential for formation of a nuclear egress complex.

Results: Mutations within a globular domain interfere with the function of pUL50.

Conclusion: Residues E56 and Y57 of pUL50 are essential for binding to pUL53.

Significance: Identification of the mode of important viral protein interactions promotes the development of novel antiviral strategies.

Summary

Herpesviral capsids are assembled in the host cell nucleus and are subsequently translocated to the cytoplasm. During this process, it has been demonstrated that the human cytomegalovirus (HCMV) proteins pUL50 and pUL53 interact and form, together with other viral and cellular proteins, the nuclear egress complex at the nuclear envelope. In this study, we provide evidence that specific residues of a conserved N-terminal region of pUL50 determine its intranuclear interaction with pUL53. In silico evaluation and biophysical analyses suggested that the conserved region forms a regular secondary structure adopting a globular fold. Importantly, site-directed replacement of individual amino acids by alanine indicated a strong functional influence of specific residues inside this globular domain. In particular, mutation of the widely conserved residues E56 or Y57 led to a loss of interaction with pUL53. Consistent with the loss of binding properties, mutants E56A and Y57A showed a defective function in the recruitment of pUL53 to the nuclear envelope in expression plasmid-transfected and HCMV-infected cells. In addition, in silico analysis suggested that residues 3-20 form an amphipathic α-helix which appears to be conserved among Herpesviridae. Point mutants revealed rather a structural role of this N-terminal α-helix for pUL50 stability than playing a direct role for the binding of pUL53. In contrast, the central part of the globular domain including E56 and Y57 is directly responsible for the functional interaction with pUL53 and thus determines formation of the basic nuclear egress complex.

Introduction

Human cytomegalovirus (HCMV) is the type species of β-Herpesvirinae (family Herpesviridae) and represents a ubiquitous, clinically highly important human pathogen. HCMV infection is mostly associated with mild pathogenesis in immunocompetent hosts, but may cause severe systemic or even life-threatening disease in immunosuppressed hosts and prenatally infected children (1). As characteristic for most DNA viruses, HCMV replicates genomes in the host cell nucleus. Thereafter, preformed viral capsids which are packaged with genomic DNA have to be transported to the cytoplasm for final maturation and release of infectious virions. Due to the large size of HCMV capsids (~130 nm) (2), these cannot be transported through the nuclear pore complex (~40 nm) (3). The currently accepted model for nuclear egress of HCMV and other herpesviruses is based on a complex envelopment/de-envelopment/re-envelopment process (4-6). Hereby, a transient primary envelopment is achieved by budding through the inner nuclear membrane (INM) (4,7,8). Before herpesviral capsids gain access to the INM, however, the proteinaceous network of the

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nuclear lamina provides a major obstacle. Thus, the locally restricted destabilization of the nuclear lamina is a rate-limiting step during the viral replication process (10). As reported previously, the HCMV-specific nuclear egress complex (NEC) is composed of viral and cellular proteins, in particular protein kinases with the capacity to induce destabilization of the nuclear lamina (11-14). Viral protein kinase pUL97 and cellular protein kinase C (PKC) play important roles by phosphorylating several types of nuclear lamins. In lamin A/C, a phosphorylation-dependent binding motif for the peptidyl-prolyl cis/trans-isomerase Pin1 leads to the local recruitment of Pin1, which is suggestive to contribute to the reorganization of the nuclear lamina (12). As two basic viral proteins, essentially involved in the complex formation of a functional NEC, pUL50 and pUL53 have been characterized in their interaction properties (11,15-17). The question if both proteins form a stable or transient complex, the latter possibly characterized by a dynamic change in composition over the time of infection, remains unclear so far. However, pUL50 and pUL53 directly interact with each other to form heterodimers (15,17). Furthermore, pUL50 possesses interaction domains for other NEC proteins, such as PKC and p32 (11,15). The region of pUL50 responsible for pUL53 interaction was previously attributed to the N-terminal amino acids 1 to 250 (11). In the case of pUL53, a short region comprising amino acids 50 to 84 was identified as the site required for binding to pUL50 (17) (Fig. 1A). The interaction of pUL50 with pUL53 appears as a prerequisite for a powerful relocalization activity of pUL50: full-length pUL50 relocates pUL53 towards a colocalization at the nuclear rim, while evenly distributed throughout the nucleus in the absence of pUL50 (or the presence of mutant pUL50 that is lacking its C-terminal trans-membrane domain) (15,16). Proteins with homology to HCMV pUL50 and pUL53 are present in members of all three herpesvirus subfamilies and a pairwise interaction between the respective proteins could be demonstrated for various examples (20-23). In this report, we identified a globular domain as well as an amphipathic helix within the N-terminus of pUL50 and we investigated the impact of these regions on the intranuclear interaction with pUL53. Experimental evidence is provided demonstrating the functional importance of individual residues inside the globular domain. In addition, novel computational data for pUL50 is presented supporting our current model on combined structure-function relationship. A comparison with the respective elements in homologous proteins of other herpesviruses is discussed.

Experimental Procedures

Eukaryotic expression plasmids–Plasmids were constructed for the recombinant expression of viral or cellular proteins in human cells. Expression constructs coding for truncation mutants of HCMV-encoded pUL50 were generated by PCR amplification of the pUL50 open reading frame. N-terminal deletion (i.e. encoded amino acids 5-397, 10-397 or 15-397) and C-terminal deletion mutants of pUL50 (i.e. encoded amino acids 1-181, 1-150, 1-130, 1-100 or 1-70) were generated by cloning of PCR products. Standard PCR amplification was performed using the template pcDNA-UL50-HA (15) with oligonucleotide primers purchased from Biomers (Ulm, Germany); sequences of oligonucleotides are given in Table S1. After cleavage with EcoRI/XhoI or HindIII/BamHI, respectively, PCR products were inserted into the vector pcDNA3.1 (Invitrogen) resulting in expression constructs coding for N-terminal deletion mutants of pUL50 fused C-terminally to a hemagglutinin (HA)-tag or inserted into the vector pcGFP-N1 (Clontech) resulting in expression constructs coding for C-terminal deletion mutants of pUL50 fused C-terminally to the green fluorescent protein (GFP). For generating constructs coding for fragments of pUL50 (i.e. encoded amino acids 1-358, 1-205, 236-358 or 10-169) fused to GFP and βgal, PCR amplification was performed as described above. Subsequently, PCR fragments were cleaved with NheI/XbaI and inserted into pHM830 (Addgene). In addition, expression constructs coding for mutant pUL50 carrying amino acid exchanges to alanine (single mutants D10A, L11A, V12A, Q13A, T15A, I18A, K20A, E56A, Y57A, N76A, G78A, P90A, L116A, K123A, R136A, G152A and P153A; double mutants D10A/Q13A and L11A/V12A) were generated by site-directed mutagenesis using pcDNA-UL50-HA (15) as template and oligonucleotide primers with nucleotides differing from the wild-type sequence (Table S2). The expression constructs pcDNA-UL50-F, pcDNA-UL53-F, pcDNA-PKCa-F as well as plasmids encoding N-terminal deletion (i.e. encoded amino acids 20-397, 40-397, 70-397, 100-397 or 150-397) and C-terminal deletion mutants of pUL50 (i.e. encoded amino acids 1-340, 1-310, 1-280 or 1-250) have been described previously (11,15).
Construct pHM830 (Addgene), expressing a GFP-βgal fusion protein, was used as a vector for protein-protein interaction experiments by communoprecipitation (CoIP).

Indirect immunofluorescence assay and confocal laser scanning microscopy—HeLa cells were cultivated and grown on coverslips for transient transfection by the use of Lipofectamine 2000 (Invitrogen) under previously described conditions (11). Primary human foreskin fibroblasts (HFFs) were used for combined transfection-infection experiments. First, HFFs were transfected with expression plasmids by the use of Fugene HD (Roche) according to the manufacturer’s protocol. One day later, transfected HFFs were infected with HCMV laboratory strain AD169 at a multiplicity of infection of 1.0. At two days post-transfection (transfected HeLa cells) or at three days post-infection (transfected-infected HFFs), respectively, cells were fixed and permeabilized following indirect immunofluorescence staining as described previously (11). The mouse monoclonal antibody (mAb) anti-FLAG (M2; Sigma) and the rabbit polyclonal antisera (pAb) anti-hemagglutinin (HA.11; Covance Inc.) were used to detect the transiently expressed tagged proteins. The mouse pAb anti-UL53 was used for the detection of pUL53 of HCMV (kindly provided by Dr. P. Dal Monte, Bologna, Italy). Secondary antibodies used for double staining were Alexa Flour® 488-conjugated goat anti-rabbit IgG (H+L) and Alexa Flour® 555-conjugated goat anti-mouse IgG (H+L; New England Biolabs GmbH). Images were acquired using a Leica TCS SP5 confocal laser scanning microscope equipped with a 63x HCX PL APO CS oil immersion objective lens (Leica). Images were analyzed and signal intensities were quantified using LAS AF software (Leica).

Coimmunoprecipitation (CoIP) assay—293T cells were cultivated and transfected by the use of polyethylenimine-DNA complexes as described previously (11,24). Two days post-transfection, immunoprecipitation was performed under previously described conditions (11) using 2 µl of mouse mAb anti-FLAG (M2; Sigma), mouse mAb anti-HA (12CA5; Roche) or mouse mAb anti-GFP (clones 7.1/13.1; Roche), respectively. Coimmunoprecipitation samples and expression controls taken prior to the addition of the CoIP antibody were subjected to standard Western blot analysis using mouse mAbs anti-FLAG (M2; Sigma), anti-HA (12CA5; Roche), anti-GFP (clones 7.1/13.1; Roche), anti-β-actin (AC-15; Sigma) or rabbit pAb anti-HA (HA.11; Covance Inc.).

Prokaryotic expression and purification of pUL50(1-181)—The N-terminal fragment of pUL50 containing residues 1-181 was expressed in Escherichia coli BL21(DE3) as a SUMO-fusion protein using the pE-SUMOpro system (LifeSensors Inc.) containing a His-tag at the N-terminus. Bacterial cells were grown in LB medium in the presence of 100 µg/ml ampicillin at 28 °C to an OD600 of 0.5-0.6, before the temperature was lowered to 20 °C and protein expression was induced with 0.25 mM isopropyl β-d-thiogalactopyranoside (IPTG). The protein was expressed overnight, bacterial cells were harvested by centrifugation and resuspended in His-trap binding buffer (50 mM phosphate buffer (pH 7.4), 300 mM NaCl, 30 mM imidazole, 3 mM DTT) containing protease inhibitors and disrupted by sonication. After centrifugation at 100,000 g for 1 h at 4 °C the supernatant containing His-SUMO-pUL50(1-181) was purified by affinity chromatography using a His-Trap column (GE healthcare). After the first His-trap column, the SUMO-tag was cleaved using 5 U of SUMO protease/100 µg of fusion protein for 5-7 days at 4 °C in 20 mM Tris (pH 8.0), 150 mM NaCl and 3 mM DTT. Both the SUMO-tag and SUMO-protease were removed via a second His-trap chromatography. pUL50(1-181) was further purified by size exclusion chromatography and concentrated to 10 mg/ml in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 3 mM DTT. Typically, 1 l of bacterial culture yielded about 2.5 mg of pure protein.

Circular dichroism spectroscopy and thermal denaturation analysis—Circular dichroism (CD) measurements were performed at 20 °C using a Jasco J-810 spectropolarimeter (JASCO International Co.) and a cuvette with 0.1 cm path length. All experiments were performed in 20 mM potassium phosphate buffer (pH 7.6) at a protein concentration of 0.25 mg/ml. Spectra were registered between 185 and 260 nm and corrected for the contributions from the phosphate buffer. Spectra were accumulated eight times with a band width of 1.0 nm and scan speed of 20 nm/min, time response of 1 s and data pitch 0.1 nm. CD spectra were analyzed using the deconvolution program CDSSTR within the Jasco CDPro program. Heat-induced unfolding transitions were also measured under similar buffer conditions and protein concentration. Ellipticity values were recorded at 222 nm by raising the temperature from 20 to 96 °C.
°C using a PTC 423S/15 Peltier element in 1 °C steps and denaturation analysis was performed using the Jasco Spectra analysis program.

**Protein sequence alignment**—Using the ClustalW algorithm (25) by AlignX (component of Vector NTI Advance 9.1.0; Invitrogen), multiple alignments of the full amino acid sequences of a total of 11 members of the UL34 family were performed. The sequences were selected from the NCBI database Entrez Protein (http://www.ncbi.nlm.nih.gov/protein): UL34 of HSV1 strain 17 (accession number: P10218); UL34 of HSV2 strain HG52 (P89457); UL34 PrV strain Kaplan (Q9ICS7); ORF24 of VZV strain Dumas (P09280); pUL50 of HCMV strain AD169 (P16791); pM50 of MCMV strain Smith (YP_214063); U34 of HHV-6A strain Uganda (P52465); U34 of HHV-6B strain HST (Q9QJ35); U34 of HHV-7 strain JI (P52466); BFRF1 of EBV strain B95-8 (P03185); ORF67 of HHV-8 strain GK18 (Q76RF3).

**Secondary structure prediction**—A secondary structure consensus prediction of HCMV-encoded pUL50 and homologous proteins was performed at the NPS@ server (network protein structure consensus prediction of HCMV-UL34 of HSV2 strain HG52; UL34 PrV strain Kaplan (Q9ICS7); ORF24 of VZV strain Dumas (P09280); pUL50 of HCMV strain AD169 (P16791); pM50 of MCMV strain Smith (YP_214063); U34 of HHV-6A strain Uganda (P52465); U34 of HHV-6B strain HST (Q9QJ35); U34 of HHV-7 strain JI (P52466); BFRF1 of EBV strain B95-8 (P03185); ORF67 of HHV-8 strain GK18 (Q76RF3).

**Results**

The N-terminal region 10-169 of pUL50 is responsible for pUL53 binding—Our previous studies demonstrated a high-affinity interaction between HCMV pUL50 and pUL53, both harboring several conserved functional elements and domains (Fig. 1A and B). In order to narrow down the domain responsible for pUL53 interaction, C- and N-terminal truncation mutants of pUL50 were generated and fused to a C-terminal coding region of the green fluorescent protein (GFP) or hemagglutinin (HA)-tag, respectively. First, pUL50 truncation mutants were tested in their ability to interact with pUL53 in a coimmunoprecipitation (CoIP) assay (Fig. 2). With C-terminal truncations, specifically the construct comprising amino acids 1 to 181 of pUL50 was coimmunoprecipitated by pUL53 (Fig. 2A, CoIP, lane 4), while further C-terminal deletions of pUL50 abrogated the interaction (lanes 5-8). As an additional important feature, truncation mutants 1-181 and 1-150 of pUL50 were expressed at high levels (Fig. 2A, expression controls, lanes 4-5 and 9-10), while truncations of more than N-terminal 150 amino acids showed decreased expression levels (lanes 6-8 and 11-13). It should be stressed that the interaction-negative mutant 1-150 (Fig. 2A, lanes 5 and 10) was expressed to similar levels as the interaction-positive mutant 1-181 (lanes 4 and 9), thus illustrating the reliability of this experiment even under variable expression situations (also note the reliable amounts of proteins present in the immunoprecipitates; Fig. 2A, precipitation controls). In addition, any unspecific binding of the GFP-tag was excluded by control settings, i.e. using GFP-tagged PKCα (Fig. 2A, lane 2) and empty-vector controls (lanes 9-13). With N-terminal truncations, deletions up to amino acid 10 were tolerable for interaction (Fig. 2B, CoIP, lanes 4-5), though the CoIP signal was weaker compared to full-length pUL50 (lane 3). Notably, the expression was lower for mutants 15-397 and 20-397 (Fig. 2B, expression controls, lanes 6-7) than for mutants 5-397 and 10-397 (lanes 4-5) possibly indicating a stabilizing role of amino acids 11 to 14. Here again, the reliability of the experiment was provided by an additional staining of the CoIP blot with a pUL50-specific antibody demonstrating equal amounts of precipitated proteins (Fig. 2B, precipitation controls, lanes 3-7). Thus, CoIP analyses with these N- and C-terminal truncation mutants suggest that amino acids 10 to 181 of pUL50 are sufficient for binding to pUL53.

To further examine the pUL53 interaction domain, constructs were generated which should transfer the interaction region of pUL50 into a GFP-βgal fusion protein. These constructs were also analyzed concerning their ability to interact with pUL53 (Fig. 2C). In contrast to GFP-βgal alone (vector; lane 1) or a fusion protein containing a putative non-interacting pUL50 sequence (i.e. amino acids 236-358; lane 4), fusion proteins which comprise amino acids 1-358, 1-204 or 10-169 were all able to coimmunoprecipitate pUL53 (lanes 2-3, 5). Notably, the CoIP signal in the case of 10-169 was weaker compared to 1-358 and 1-204. This demonstrates that a minimal interaction domain
The interaction of pUL53-pUL50 is essential for the relocalization of pUL53 to the nuclear rim—in a next step, pUL50 truncation mutants were analyzed with respect to their ability to recruit pUL53 to the nuclear envelope using immunofluorescence analysis and confocal laser-scanning microscopy (Fig. 3). Notably, all analyzed C-terminal truncation mutants showed a diffuse nuclear localization due to the lack of the transmembrane domain and, therefore, were not able to recruit pUL53 to the nuclear rim (summarized schematically in Fig. 3B). In the cases of N-terminal truncation, all mutants expressed alone were localized at the nuclear rim and cytoplasmic membranes similar to full-length pUL50 (data not shown). When coexpressed with pUL53, the interaction-competent mutant pUL50(5-397)-HA showed a perfect colocalization with pUL53 at the nuclear rim (Fig. 3A, panels i-m; compare full-length in panels e-h), while no recruitment was noted for non-interacting mutant pUL50(20-397)-HA (panels n-q). This correlation between detectable pUL50-pUL53 interaction and pUL53 relocalization was a general feature of all mutants analyzed, i.e. pUL53 recruitment to the nuclear rim was restricted to those mutants of pUL50 which were positive for interaction in the CoIP analysis (Fig. 3B) (11). In addition, coexpression experiments demonstrated that the complete nuclear rim localization of pUL50 itself was also restricted to those versions of pUL50 that are interaction-competent for pUL53 (Fig. 3A, panels e-m compared to panels n-q). These findings underline that the interaction pUL50-pUL53 is a prerequisite for the correct nuclear localization of both proteins. Recently, a similar finding for the homologous proteins of HSV-1 was published by Roller et al. (34), thus suggesting that this functional aspect may be conserved amongst herpesviruses.

Comparison between conserved regions using sequence alignments of pUL50 homologs—Multiple sequence alignments of pUL50 and homologous proteins of human herpesviruses (i.e. HSV-1, HSV-2, VZV, HHV-6A, HHV-6B, HHV-7, EBV and HHV-8) and animal herpesviruses (i.e. MCMV and PrV) were generated to address the question of a conserved mode of interaction (Fig. 4A). Interestingly, the N-terminus is the most conserved part of pUL50 (Fig. 1B). In particular, the consensus sequence within this region revealed a high degree of conservation of the corresponding residues. Within this conserved part, more than 30% (β-Herpesvirinae) or 3% (Herpesviridae) of the residues were identical and were found in all analyzed sequences. Thus, the N-terminus of pUL50 and homologous proteins is relatively well-conserved and, in case of pUL50, contains the pUL53 interaction region. We next addressed the issue, which structural features determine the interaction properties of the N-terminal part of pUL50.

Structural information on pUL50 determined by bioinformatic and biophysical evaluation—A modeling approach derived from the secondary structure prediction of the HCMV-encoded pUL50 amino acid sequence (11) suggested that two distinct structural elements could be present in the N-terminal region of amino acids 1-181 (Fig. 1B). These elements, i.e. an N-terminal α-helix (3-20) and a globular domain (45-181), appeared to have an impact on the interaction properties of pUL50, based on the finding that truncation of either of these two elements was incompatible with pUL53 binding. However, the use of novel globularity scales for globular domain prediction suggested that the globular domain comprises amino acids 1 to 209 indicating that the N-terminal α-helix is rather a part of the globular domain than a distinct structural element (data not shown). In order to confirm this new concept, pUL50(1-181) was expressed in Escherichia coli, affinity purified via an N-terminal His-tag and analyzed by circular dichroism (CD) spectroscopy. Far-UV-CD spectra of purified pUL50(1-181) suggested a folded protein with a mixed α-helix and β-sheet fold (Fig. 1C). Importantly, the temperature-induced change of ellipticity at 222 nm reveals a highly cooperative unfolding transition pointing to the existence of a single globular fold within the N-terminal residues 1-181 of pUL50 (Fig. 1D). Thus, the globular domain appears to comprise at least residues 1-181 including the α-helix in the N-terminus of pUL50.

The relatively high degree of sequence conservation in the N-terminus of pUL50 and homologs, i.e. α-herpesviral (HSV-1 and PrV UL34), β-herpesviral (MCMV pM50) and γ-herpesviral (EBV BFRF1) homologs, allowed a comparative setting to predict their tendency to
adopt a common regular secondary structure in the corresponding regions. Interestingly, N-terminal α-helices were predicted in all cases (Fig. S1). Closer inspection of side chain properties disclosed segregation of hydrophobic and polar residues opposed to each of the two faces of these α-helices (Fig. 4B), which is characteristic for amphipathic helices (37). The mean helical hydrophobic moment µH (representing a degree of amphiphilicity of helices) (38) was in a similar range for all analyzed proteins (i.e. µH of ~0.4 for MCMV p50 and ~0.65 for EBV BFRF1).

Specific residues within the globular domain of pUL50 are responsible for the functional interaction with pUL53—In order to investigate which residues within the interaction domain are directly involved in binding to pUL53, we performed alanine replacement mutations of single amino acids. First, a possible involvement of the predicted N-terminal amphipathic helix was analyzed. Region 10-20 was subjected to mutation, since deletion of these residues resulted in the complete loss of interaction with pUL53 (Fig. 2B). In particular, we produced three mutants with replacements on the predicted polar side (i.e. D10A, Q13A and K20A) and four mutants with replacements on the predicted hydrophobic side (i.e. L11A, V12A, T15A and I18A) (Fig. 4C). Additionally, two double-mutants were generated with double-replacements on either side (i.e. the polar, D10A/Q13A, or hydrophobic side, L11A/V12A, respectively). These point mutants were used for CoIP and immunofluorescence analyses to determine their interaction and recruitment properties (Fig. 5A and Fig. 6A). CoIP analysis demonstrated that coimmunoprecipitation of pUL53 was positive for the four mutants with replacements on the polar side (Fig. 5A, CoIP, lanes 3-5 and 9) and the two hydrophobic side mutants V12A and T15A (lanes 6-7). A significantly reduced CoIP signal was detected for mutant I18A (Fig. 5A, CoIP, lane 8). Importantly, the Western blot expression controls revealed a decreased expression level for mutant I18A (Fig. 5A, expression controls, lane 8; compare wild-type in lane 2). Staining of β-actin served as a loading control (Fig. 5A, expression controls, bottom panel). Interestingly, this imbalance in expression levels was mostly compensated during the CoIP procedure by the limited binding capacity of the precipitation antibody, finally leading to an immunoprecipitation of all mutants quantitatively similar to wild-type pUL50 (Fig. 5A, precipitation controls, lanes 2-9). Notably, no CoIP signal was observed for mutants with hydrophobic side mutation L11 (Fig. S2A). However, the loss of interaction properties of single-mutant L11A and double-mutant L11A/V12A was rather a result of inaccurate protein folding than caused by a direct involvement of L11 in binding to pUL53. This was concluded from three observations: mutants L11A and L11A/V12A were expressed at significant lower levels as wild-type pUL50 (Fig. S2A; data not shown); L11A showed defective intracellular localization in transfected HeLa cells (i.e. L11A is not only localized at the INM but accumulates at aggregates in the cytoplasm; Fig. S2B); L11A was not able to interact with PKCα as another pUL50 binding partner (Fig. S2C). In case of the stable mutants of the amphiphatic helix, analysis of recruitment properties produced positive results consistent with the CoIP findings with exception of mutant I18A that showed an intermediate phenotype: As expected, all pUL53-interacting mutants were able to recruit pUL53 to the nuclear envelope (see examples in Fig. 6A; summarized in Table 1). Surprisingly, mutant I18A was not impaired in its recruitment properties (Fig. 6A, panels l-p) even though its ability to interact with pUL53 was reduced in CoIP assay (Fig. 5A). The latter observation suggested that mutation of specific residues on the hydrophobic side might affect the folding of pUL50 but that the amphipathic helix is not directly involved in the functional interaction with pUL53.

In a second step, we concentrated on the most conserved part of the globular domain to identify a region of pUL50 which is directly involved in the binding of pUL53. Therefore, six residues within the central part of the globular domain, fully conserved among all analyzed sequences (i.e. E56, N76, G78, P90, K123 and R136), as well as four residues conserved only among α- and/or β-Herpesvirinae (i.e. Y57, L116, G152 and P153) were replaced by alanine. Analyzing the interaction and recruitment properties of these mutants, CoIP and relocation analyses produced consistent results. In the CoIP analysis, mutants E56A and Y57A were negative for a wild-type-like, efficient interaction with pUL53 but retained a marginal level of residual binding activity (Fig. 5B, CoIP, lanes 1-2). Moreover, all these mutants of the central part of the globular domain showed mostly normal, wild-type-like expression levels (Fig. 5B, expression controls). As an exception in this regard, the slightly reduced protein levels detected for the pUL53 non-interacting point mutants E56A and Y57A
might result from the lack of a stabilizing binding to pUL53, which might indicate the importance of interaction for protein stability of both partners (Fig. 5B, expression controls, lanes 1-2 compared to lanes 3-10). However, in clear contrast to I18A, mutants E56A and Y57A had lost the ability to relocalize pUL53 to the nuclear rim (Fig. 6B, panels a-k), whereas other mutants of the central part of the globular domain were not impaired in this activity (see examples in Fig. 6B). A summary of the combined interaction and recruitment analyses is depicted in Table 1.

To exclude that the reduced ability of mutants E56A and Y57A to interact with pUL53 was caused by general defects in protein folding, mutants were analyzed in regard of their interaction with PKCα as another known pUL50 binding partner (Fig. S2C). Interestingly, CoIP analysis demonstrated that mutations E56A and Y57A were tolerable for interaction with PKCα (Fig. S2C, CoIP, lanes 5-6), although the CoIP signal was weaker compared to wild-type pUL50 (lane 2). Notably, pUL53 and PKCα interaction regions within pUL50 are partly overlapping (11). This indicates that pUL50 mutants E56A and Y57A had lost the pUL53 interaction phenotype most probably as a direct consequence of the amino acid replacements but not an overall loss of protein folding. This assumption was confirmed by the fact, that unstable mutant L11A not only showed a loss of pUL53 binding but also PKCα binding (as represented by a very low CoIP signal at background levels; Fig. S2C, CoIP, lanes 1 and 3). The reliability of this experiment was provided by additional immunostaining of the CoIP blot with a pUL50-specific antibody which demonstrated equal amounts of pUL50 mutants in the precipitates (Fig. S2C, precipitation controls, lanes 2-6). In conclusion, among all analyzed stable mutants, only E56A and Y57A were impaired in both, their pUL53 interaction and recruitment properties suggesting that the central part of the globular domain (including E56 and Y57) is directly involved in pUL53 binding.

**Residues E56 and Y57 of pUL50 are essential for colocalization with pUL53 in HCMV-infected cells**—In order to confirm the importance of residues E56 and Y57 of pUL50 for the functional interaction with pUL53 in the native environment, combined transfection-infection experiments were performed. Therefore, primary human foreskin fibroblasts (HFFs) were individually transfected with expression constructs coding for HA-tagged wild-type pUL50 or the non-interacting mutants E56A and Y57A. The transfected HFFs were additionally infected with HCMV strain AD169. Three days post-infection, the transfected-infected HFFs were used for immunofluorescence analysis and analyzed by confocal laser-scanning microscopy to detect viral proteins in HCMV-infected cells. Thus, the experimental setting allowed a mixed expression of native viral proteins and transiently coexpressed pUL50-HA (Fig. 7). Notably, in transfected HeLa cells overexpressing pUL53 without any other viral proteins, pUL53 shows a diffuse nuclear localization (15). However, in HFFs infected with HCMV, pUL53 is incorporated into the NEC (6,11,14) which is represented here by an speckled aggregation of pUL53 at specific sites of the nuclear envelope (Fig. 7A, panels e, p and u). In the combined setting of transfected-infected cells, transiently expressed pUL50-HA colocalized with accumulations of the virus-produced pUL53 (Fig. 7A, panels a-e, filled arrowheads). In contrast, the non-interacting mutants E56A and Y57A were distributed homogenously at the nuclear envelope of infected cells (Fig. 7A, panels l-u). As expected, pUL53 still accumulated at distinct sites of the nuclear envelope in these cells (Fig. 7A, panels p and u, open arrowheads). This was explained by the fact that virus-produced wild-type pUL50 (unstained) was also present, but the pUL50 mutants were missing in these speckled sites (panels p and u). Notably, the homogenous distribution of E56A and Y57A in HCMV-infected cells was similar to the localization of these mutants (data not shown) or wild-type pUL50-HA in uninfected cells (Fig. 7A, panels f-k). In addition, sequential optical planes in the z axis (z stacks) were acquired from these samples and signal intensities were quantified (Fig. 7B-D). Thereby, single focal planes at the bottom of the infected cell nuclei were used (Fig. 7B-D, indicated by dashed lines in xz and yz). Consistent with images taken from the center of the nuclei (Fig. 7A), transiently expressed pUL50-HA and virus-produced pUL53 accumulated at distinct sites of the nuclear envelope (Fig. 7B, inset in xy). Quantification of signal intensities demonstrated that pUL50-HA and pUL53 colocalize at these sites (Fig. 7B, graph; indicated by covering peaks of pixel intensities). As expected, signal intensities of mutants E56A (Fig. 7C) and Y57A (Fig. 7D) remained at a bottom line and did not show any peak of colocalization, as also represented by a diffuse green staining at the bottom of the nuclei.
(Fig. 7C-D, inset in xy). In summary, we concluded that amino acids E56 and Y57 of pUL50 are essential for the functional interaction with pUL53 in the native environment of HCMV-infected cells.

Discussion

Multiple activities of pUL50 and homologs during virus replication—The conserved herpesviral proteins pUL50 and pUL53 play important roles during the nuclear egress of viral capsids. For β-herpesviruses, a focus of investigations was directed to the initial local disruption of the nuclear lamina which allows capsids access to the INM. In particular, pUL50 and pUL53 represent the core of a viral-cellular NEC which mediates the recruitment of protein kinases essential for the phosphorylation-dependent disassembly of the nuclear lamina (11-14,16). For the α-herpesviral homologs of pUL50 and pUL53, additional evidence suggested that both proteins function at later stages of nuclear egress including capsid docking at the INM, curvature of the membrane around capsids and de-envelopment at the outer nuclear membrane. Furthermore, a putative role for cell-to-cell spread was discussed for the pUL50 homolog of HSV-1 (UL34) (14,19,34,39-42). All these functions rely on the pUL50-pUL53 interaction as an essential step of complex formation at the nuclear envelope. Thus, the identification of essential structural elements required for interaction has been aspired.

Conserved residues E56 and Y57 of pUL50 are essential for NEC formation—Data in this study suggest that the complete N-terminus of pUL50 (aa 1-181) is necessary for efficient binding to pUL53 (Fig. 2 and Fig. 3). Nevertheless, a domain swapping experiment demonstrated a transfer of amino acids 10 to 169 to a large GFP-βgal fusion protein being sufficient to confer pUL53 interaction. Recently, an N-terminal α-helix (aa 3-20) and a putative globular domain, including at least residues 45-181, were identified by bioinformatic analyses in this region (11). Notably, novel data derived from thermal denaturation experiments for pUL50 indicate that the complete N-terminus adopts a globular fold (i.e. aa 1-209) and there is no evidence that the N-terminal α-helix represents an independent structural element. A short region within the globular domain including highly conserved E56 and Y57 appears to be required for high-affinity binding to pUL53, since amino acid exchanges of both residues to alanine abrogated pUL50-pUL53 coimmunoprecipitation (Fig. 5). This suggests that E56 and Y57 may be exposed at the surface of the globular domain. General defects in protein folding produced by E56A and Y57A appear not to be likely since the mutants (Fig. 5, Fig. 6 and Fig. S2C) (i) are stably expressed to similar levels compared to wild-type pUL50, (ii) show a regular localization at the nuclear envelope, and (iii) are still able to bind PKCα. In addition, residues E56 and Y57 of pUL50 were not only proven to be important for the physical interaction with pUL53, but also for the recruitment of pUL53 to the HCMV-specific NEC: in cotransfected cells, the non-interacting mutants E56A and Y57A failed to recruit pUL53 to the nuclear envelope (Fig. 6); in the transfection-infection setting, mutants E56A and Y57A were not incorporated into the HCMV-specific NEC (Fig. 7). In conclusion, the central part of the globular domain including E56 and Y57 mediates binding to pUL53 and is therefore essential for pUL50 functionality.

The mode of interaction between pUL50 and pUL53 is conserved among cytomegaloviruses—The general importance of E56 and Y57 was illustrated by Bubeck et al. (36), demonstrating that the corresponding residues in the MCMV protein pM50 were similarly essential for coimmunoprecipitation of pM53. Surprisingly, despite of a high conservation of these residues among Herpesviridae, the pUL50-homologous region of the HSV-1 UL34 protein proved to be dispensable for UL31 interaction (35). However, the residues homologous to E56 and Y57 (HSV-1 UL34 residue E67 and Y68) are critical for virus replication in HSV-1 (19,43). In particular, a charged cluster mutant of HSV-1 UL34, CL06, also containing E67A, was not able to complement infection with a UL34-null virus. Surprisingly, CL06 failed to localize to the INM but still colocalized with UL31 suggesting some residual activity in UL34-UL31 interaction (43). Moreover, residue Y68 of UL34 showed similar functional importance, since mutation Y68A produced a major defect in virus replication, an impairment in nuclear capsid egress and defects in cell-to-cell spread. Interestingly, indirect evidence suggested that mutation Y68A did not interfere with UL31 interaction (19). Taken together, we conclude that the mode of interaction of viral egress proteins is conserved among cytomegaloviruses (dependent on the highly conserved residues E56 and Y57) but differs in several aspects from HSV-1 and possibly other herpesviruses.
Importance of the N-terminal amphipathic helix of pUL50—The presence of an amphipathic helix in the extreme N-terminus of pUL50 was predicted by bioinformatics analyses (also including conservation amongst homologous proteins of Herpesviridae). This prediction proved to be compatible with a functional analysis of replacement mutants. Residue I18 appeared to be functionally important as it is situated on the hydrophobic face of the putative amphipathic helix of pUL50. In our experimental evaluation, mutant I18A showed an impairment of binding to pUL53 but still recruited pUL53 to the nuclear envelope. In accordance with a reduced expression level in transfection experiments, this intermediate phenotype might rather result from inaccurate protein folding than from direct involvement in pUL53 binding. Interestingly, mutation of amino acid L11, positioned adjacent to I18 (Fig. 4B-C), resulted in a more drastic phenotype. Mutant L11A had completely lost its functionality in interaction (neither binding to pUL53 nor to PKCα) and recruitment of pUL53 (Fig. S2), most probably due to a loss of correct folding and stability. Taken together, the hydrophobic side of the amphipathic helix appears to possess a stabilizing function for pUL50, but is not directly involved in the interaction with pUL53. Nevertheless, it is tempting to speculate that the conserved N-terminal amphipathic helix is functionally critical for pUL50 and its herpesviral homologs. The charged cluster mutant CL02 of HSV-1 UL34 carries mutations in the predicted helix (Fig. S1; QRIRL>QAIAL) and exhibits a defect in virus replication (43). Interestingly, the function of UL34 to support INM curvature around capsids was mapped to its N-terminus adjacent to the putative α-helix (42).

The functional importance of the conserved N-terminal domain of pUL50—Summarized, we conclude on the functional importance of the pUL50 N-terminus (aa 1-181) as follows: (i) the globular domain (including the amphipathic helix) determines interaction with pUL53, (ii) the amphipathic helix is not directly involved in binding but attributes to protein stability and folding, (iii) residues E56 and Y57 are essential for pUL53 interaction and recruitment to the nuclear envelope. Thus, the residues essential for pUL50-pUL53 interaction are determinants of functional association of the entire NEC. This crucial contact point for multiple NEC interactions may represent a highly interesting target for future antiviral strategies.

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Footnotes

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Keywords—Human cytomegalovirus; Nuclear capsid egress; Nuclear lamina-associated proteins; Nuclear egress complex

Abbreviations—aa, amino acids; CoIP, coimmunoprecipitation; CD, circular dichroism; CR, conserved region; DAPI, 4’,6-diamidino-2-phenylindole; EBV, Epstein-Barr virus; GFP, green fluorescent protein; HA, hemagglutinin; HCMV, human cytomegalovirus; HHV, human herpes virus; HSV, herpes simplex virus; INM, inner nuclear membrane; mAb, monoclonal antibody; MCMV, murine cytomegalovirus; NEC, nuclear egress complex; NLS, nuclear localization signal; pAb, polyclonal antibody; PKC, protein kinase C; PrV, pseudorabies virus; RFP, red fluorescent protein; VZV, varicella-zoster virus; Wb, Western blot.
Figure legends

FIGURE 1. Structural elements, conserved regions and functional domains of the HCMV-encoded nuclear egress proteins pUL50 and pUL53. A, pUL53 contains a putative N-terminal nuclear localization signal (NLS; green box) (15) and a calculated globular domain (black box) in its central part. The four conserved regions (CR1 to CR4) identified among pUL53 homologs are depicted below (HCMV pUL53: CR1, residues 58-125; CR2, 127-160; CR3, 163-243; CR4, 254-282; initially described by Lötzerich et al.) (18). Sam et al. demonstrated that region 50 to 84 (dashed box) of pUL53 including a predicted α-helix (red box) is required for interaction with pUL50 (17). B, pUL50 contains a C-terminal transmembrane domain (blue box) (15) as well as an N-terminal globular domain (black box) (11) including a putative α-helix (red box). By analogy with pUL53, two conserved regions were identified among 11 homologs of pUL50 through multiple sequence alignment (HCMV pUL50: CR1, 1-62; CR2, 70-170). Notably, Haugo and colleagues divided the N-termini of pUL50 homologs into three conserved regions probably due to a different selection of analyzed sequences and the use of another alignment algorithm (19). The N-terminus of pUL50 was initially identified as the binding region for pUL53 (dashed box) (11,17); essential residues of this interaction region were mapped in this study (E56, Y57).

FIGURE 2. Determination of the pUL53 interaction domain within pUL50 by CoIP analyses. A and B, GFP-tagged C- or HA-tagged N-terminal truncated versions of pUL50 were transiently coexpressed in 293T cells with FLAG-tagged pUL53 or pcDNA3.1 (vector) as indicated. At 2 days post-transfection, cells were lysed and FLAG-tagged pUL53 was precipitated using mAb anti-FLAG (A), or the HA-tagged deletion mutants of pUL50 were precipitated using mAb anti-HA (B). Coimmunoprecipitates and expression control samples were subjected to Western blot (Wb) analysis using tag-specific antibodies as indicated. C, GFP-pUL50-βgal fusion proteins were transiently coexpressed with FLAG-tagged pUL53 or pHM830 (vector) as indicated. At 3 days post-transfection, CoIP was performed using mAb anti-GFP. Ig-HC, cross-reactive band for immunoglobulin heavy chain.

FIGURE 3. Interaction with pUL50 is required for pUL53 recruitment to the nuclear envelope. A, HA-tagged full-length pUL50 or N-terminal deletion mutants were transiently coexpressed with FLAG-tagged pUL53 in HeLa cells. At 2 days post-transfection, cells were subjected to indirect immunofluorescence analysis using the antibodies indicated. Samples were subsequently analyzed by confocal laser-scanning microscopy. A selection of representative images of transfected cell nuclei are shown (depicted phenotype was observed in more than 99 % of pUL50-pUL53-positive cells in each case). DAPI, 4',6-di-amidino-2-phenylindole. B, schematic summary of combined CoIP and immunofluorescence (IF) data obtained with N- and C-terminal deletion mutants of pUL50 (this study and Milbradt et al.) (11). Based on these results, the region required for binding to and recruitment of pUL53 to the nuclear envelope was determined. Structural elements within pUL50 which are present in regions important for protein function are indicated. +, CoIP positive / recruitment of pUL53 to the nuclear envelope; -, CoIP negative / no recruitment; n.d., not determined; aa, amino acids.

FIGURE 4. Putative amphipathic helices in the conserved N-terminus of pUL50 and homologous proteins of α-, β- and γ-herpesviruses. A, Multiple protein sequence alignment of pUL50 homologs. Using the ClustalW algorithm (25), multiple alignments of full amino acid sequences of pUL50 homologs within the Herpesviridae were performed. Only sections of the full sequence alignment are presented comprising the first 199 amino acids of the HCMV-encoded pUL50 protein. Amino acids corresponding to the mapped pUL53 interaction regions of HCMV pUL50 and homologous proteins of HSV1 (35) and PrV (21) are marked with colored bars. Conserved residues E56 and Y57 of MCMV (36) and HCMV pUL50 have been shown to be essential for binding to pUL53 (red framing). Alignment coloring scheme: black, non-similar residues; blue on cyan, consensus derived from a block of similar residues; black on green, consensus derived from the occurrence of greater than 50 %
of a single residue; red on yellow, consensus derived from a completely conserved residue. B and C, Putative amphipathic helices in the N-terminus of pUL50 homologs. B, the HeliQuest server (33) was used to analyze side chain properties and their distribution in the predicted α-helices. Various sequence stretches for each pUL50 homolog were analyzed which correspond to the results of the secondary structure prediction (Fig. S1). Helical wheels are depicted giving the highest mean helical hydrophobic moment (µH). Amino acids are colored according to side chain properties: yellow, hydrophobic; red/blue, acidic/basic (amount of red/blue decreases proportionally to the acidity/basicity level); grey, neutral. C, schematic presentation of side chain properties of the N-terminal α-helix formed by amino acids 3-20 of HCMV-encoded pUL50. Amino acids are illustrated in stick presentation and colored according to side chain properties as explained for (B). Only amino acids were labeled which were chosen for site-directed replacement by alanine.

FIGURE 5. Effects of mutation of specific residues of the globular domain of pUL50 on the interaction with pUL53. HA-tagged wild-type pUL50 or pUL50 point mutants were transiently coexpressed in 293T cells with FLAG-tagged pUL53. At 2 days post-transfection, CoIP analysis was performed using mAb anti-HA. Coimmunoprecipitates and expression control samples were subjected to Wb analysis using tag-specific antibodies as indicated. Ig-HC, cross-reactive band for immunoglobulin heavy chain.

FIGURE 6. Non-interacting pUL50 point mutants fail to recruit pUL53 to the nuclear envelope in transiently transfected cells. HA-tagged pUL50 point mutants of the amphipathic α-helix (A) and the central part of the globular domain (B) were transiently coexpressed in Hela cells with FLAG-tagged pUL53 as indicated. At 2 days post-transfection, cells were subjected to indirect immunofluorescence analysis using indicated antibodies. Samples were subsequently analyzed by confocal laser scanning microscopy. DAPI, 4′,6-di-amidino-2-phenylindole.

FIGURE 7. Non-interacting pUL50 point mutants fail to colocalize with pUL53 in HCMV-infected cells. HFFs were transiently transfected with constructs coding for HA-tagged wild-type pUL50 or pUL50 point mutants. One day later, the cells were additionally infected with HCMV strain AD169 at a multiplicity of infection of 1.0. At 3 days post-infection, cells were fixed and communostained with pAb anti-HA and pAb anti-UL53. (A) Localization of overexpressed pUL50-HA (wild-type or mutants) and virus-produced pUL53 in one cell. Filled arrowheads, colocalization of pUL50-HA and pUL53 at specific sites of the nuclear envelope; open arrowheads, accumulation of only pUL53 at specific sites of the nuclear envelope. (B-D) z stacks of examples of transfected-infected cells. The xz and yz axis of the cells and a single focal plane from the z axis (xy) are shown. Enlarged areas from the dashed boxes are depicted in the insets. Pixel intensities of pUL50-HA (green) and pUL53 (red) staining from along the white lines are plotted in the graphs. Dashed lines, optical section through the z stack (xy) or the focal plane (xz and yz), respectively.
Table 1: Mutation of specific residues within the N-terminal globular domain of pUL50 modulates its pUL53 interaction and recruitment to the nuclear envelope.

| pUL50 mutation | alanine replacement mutants | interaction with pUL53 | recruitment of pUL53 |
|----------------|----------------------------|-----------------------|---------------------|
| N-terminal α-helix | central part of the globular domain |
| / | D10A | V12A | Q13A | T15A | I18A | K20A | E56A | Y57A | N76A | G78A | P90A | L116A | K123A | R136A | G152A | P153A |
| + | + | + | + | + | - | + | - | - | + | + | + | + | + | + | + |

*wt, pUL50 wild-type; +, positive CoIP signal / recruitment of pUL53 to the nuclear envelope; -, substantially reduced CoIP signal / no recruitment of pUL53 to the nuclear envelope.*
Figure 1

A  HCMV pUL53

B  HCMV pUL50

C

D
Figure 3

A

B

| Recruitment of pUL53 (IF) | Interaction with pUL53 (CoIP) | pUL50 truncation mutants |
|---------------------------|------------------------------|--------------------------|
| +                         | +                            | 100 HA                   |
| -                         | +                            | 150 GFP                  |
| -                         | -                            | 130 GFP                  |
| -                         | +                            | 100 GFP                  |
| -                         | -                            | 150 GFP                  |
| -                         | +                            | 130 GFP                  |
| -                         | -                            | 150 GFP                  |
| -                         | +                            | 100 GFP                  |
| -                         | -                            | 150 GFP                  |
| n.d.                      | + GFP                        | 358 Igal                 |
| n.d.                      | + GFP                        | 358 Igal                 |
| n.d.                      | + GFP                        | 358 Igal                 |
| n.d.                      | + GFP                        | 358 Igal                 |

pUL53 interaction region: 10-169
Figure 5

A

CoIP

[pUL53-F +

IP: mAb-HA

pUL53]

WB: mAb-HA

WB: mAb-FLAG

expression control

WB: mAb-HA

WB: mAb-FLAG

WB: mAb-I-actin

B

CoIP

[pUL53-F +

IP: mAb-HA

pUL53]

WB: mAb-HA

WB: mAb-FLAG

precipitation control

WB: mAb-HA

WB: mAb-FLAG

WB: mAb-I-actin
Figure 6
SPECIFIC RESIDUES OF A CONSERVED DOMAIN IN THE N-TERMINUS OF THE HUMAN CYTOMEGALOVIRUS pUL50 PROTEIN DETERMINE ITS INTRANUCLEAR INTERACTION WITH pUL53
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