Characterization of a viral-cellular protein complex which controls the nuclear egress of human cytomegalovirus

Charakterisierung eines multimeren Proteinkomplexes, der den nukleären Kapsidexport des humanen Cytomegalovirus steuert

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A Summary

Human cytomegalovirus (HCMV) has developed a replication strategy that is well adapted to conditions of the host cell. Notably, after nuclear capsid assembly, HCMV capsids traverse the nuclear envelope for nuclear egress. In this regard, the phosphorylation-mediated disassembly of the nuclear lamina is believed to be a prerequisite for the budding of viral capsids through the nuclear membrane. Although the interplay between the nuclear lamina-associated viral proteins pUL50 and pUL53 with viral and cellular protein kinases has been considered for several years, the exact molecular events for HCMV nuclear egress were still unclear.

In this thesis, yeast two-hybrid and coimmunoprecipitation analyses were applied to detect the interaction of pUL50 with three proteins. Hereby, partly overlapping interaction domains could be identified and pUL50-associated protein complexes could be isolated using lysates of transiently transfected and HCMV-infected cells. As illustrated by immunofluorescence costaining analyses, the direct interaction of pUL50 with protein kinase C (PKC) could be confirmed and recruitment of PKC to the nuclear rim of transiently transfected cells was demonstrated. Interestingly, viral protein kinase pUL97 was also recruited by pUL50, although through a different mechanism lacking direct interaction. Experimental evidence pointed to an indirect bridging of pUL50-associated proteins mediated by the cellular adaptor p32. Combined, these data suggested the formation of a nuclear egress complex (NEC) which includes at least pUL50, pUL53, p32, the lamin B receptor and the two protein kinases pUL97 and PKC. Reports describing the ability of pUL97 and PKC to phosphorylate several types of lamins, coincided with the finding in this study that pUL97 and PKC had the potential to induce distinct, punctate lamina-depleted areas at the nuclear periphery in transiently transfected as well as HCMV-infected cells. Using a recombinant HCMV, the direct transition of GFP-labeled viral capsids through these areas could be visualized. To discover the molecular mechanism driving the NEC-mediated nuclear lamina destabilization, bioinformatical analyses were performed concerning the pUL97-dependent phosphorylation of lamin A/C at serine 22. As an important finding, phosphorylation at this specific serine generated a putative binding motif for the peptidyl-prolyl cis/trans isomerase Pin1. Interaction between Pin1 and lamin A was confirmed in HMCV-infected fibroblasts. Furthermore, the physiological localization of Pin1 was altered, leading to recruitment of Pin1 to the nuclear lamina late during infection. The local increase of Pin1 isomerase activity was suggested to promote conformational modulation of nuclear lamins. Taken together, this study strongly suggests the formation of a viral-cellular NEC which is required for the phosphorylation-triggered destabilization of the nuclear lamina during HCMV nuclear egress.
A Zusammenfassung


B Introduction

B-1 Human cytomegalovirus

Human cytomegalovirus (HCMV), also known as human herpesvirus 5 (HHV-5), belongs to the order of *Herpesvirales*, family of *Herpesviridae*. *Herpesviridae* are classified on the basis of biological properties, genome structure and sequence comparison into three subfamilies: \(\alpha\)-, \(\beta\)- and \(\gamma\)-*Herpesvirinae* (Davison A. J., 2010; Davison *et al.*, 2009). HCMV, the prototypic \(\beta\)-herpesvirus, is characterized by a restricted host range, a prolonged replication cycle in cell culture and slow progression of infection in the human host (Mocarski *et al.*, 2007). Encoding more than 200 open reading frames (ORFs), HCMV is one of the most complex pathogenic viruses. The cytomegaloviral virion is composed of a double-stranded DNA genome of about 235 kilobase pairs (kbps) enclosed in an icosahedral capsid. This capsid itself is embedded into an amorphous layer, termed the tegument, containing viral and cellular proteins, along with viral and cellular RNA. Finally, the tegument is surrounded by a host cell-derived envelope modified by inclusion of virus-encoded glycoproteins (Kaleja R. F., 2008; Mocarski *et al.*, 2007). As for all herpesviruses, primary infection with HCMV is followed by a lifelong persistence, with latent viral DNA detectable in monocyte precursors and diverse populations of tissue stromal cells (Loewendorf and Benedict, 2010). In general, HCMV lytic replication occurs in fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived macrophages (Sinzger *et al.*, 2008; Bissinger *et al.*, 2002). In contrast, *in vitro*, the only cells fully permissive for replication of HCMV laboratory strains (e.g. AD169) are human skin or lung fibroblasts, whereas clinical isolates, however, preferentially replicate on cultured endothelial cells. In fibroblasts, lytic replication induces a typical cytopathic effect (CPE) mainly characterized by cell enlargement (*cytomegaly in vivo*) and both intra- and perinuclear inclusions (Landolfo *et al.*, 2003). Due to lytic replication in different cell types *in vivo*, infectious virus is excreted into several body fluids including saliva, blood, semen, vaginal secretions and breast milk. Thus, high risk of horizontal transmission emanates from person-to-person contact, aerosol droplets, sexual contact, nursing, blood transfusion or organ transplantation (Mocarski *et al.*, 2007; Hamprecht *et al.*, 2008; Morris *et al.*, 2010). Acquired primary infection or HCMV reactivation in immunocompetent individuals is typically clinically silent, although mild mononucleosis-like symptoms can occur (Sissons and Carmichael, 2002). Rarely, severe complications of HCMV infection have been described in immunocompetent adults, with the gastrointestinal tract (colitis) and the central nervous system (meningitis, encephalitis, transverse myelitis) as the most frequently affected sites (Rafailidis *et al.*, 2008). In contrast, severe or even life-threatening pathological manifestations
are common in patients with suppressed immune functions like transplant recipients or patients infected with human immunodeficiency virus (HIV) (Steininger C., 2007; Vancikova and Dvorak, 2001). The range of severe symptoms covers hematological, hepatic and gastrointestinal abnormalities and interstitial pneumonia. Furthermore, HCMV infection was suggested to be implicated in age-associated failing systemic immunity (immunosenesence; Derhovanessian et al., 2009; Koch et al., 2007) and in proliferative diseases such as atherosclerosis (Stassen et al., 2008; Yi et al., 2008) or coronary restenosis (Corrado and Novo, 2005; Ismail et al., 1999). In addition to horizontal transmission, HCMV can also spread by intrauterine infection. HCMV is the leading viral cause of congenital anomalies in the central nervous system comprising mental retardation, sensorineural hearing loss, visual defects, seizure and epilepsy (Tsutsui Y., 2009; Revello and Gerna, 2002). Due to the high risk of infection by horizontal and vertical transmission, 50% to more than 90% of human population is HCMV seropositive, depending on the socioeconomic status of a country (Mocarski et al., 2007; Krech U., 1973). Therefore, and especially in regard to the severe complications in immunocompromised patients or newborns, there is an urgent need for existing and novel anti-HCMV drugs. To date, five antiviral compounds are approved for the worldwide treatment of HCMV infection and disease: i.e. ganciclovir, valganciclovir, cidofovir, foscarnet and fomivirsen (Schreiber et al., 2009). With the exception of fomivirsen, all approved drugs target the viral DNA polymerase pUL54. Notably, the initial phosphorylation of ganciclovir and valganciclovir by the HCMV-encoded protein kinase pUL97 is crucial for exerting their anti-cytomegaloviral activity. Cidofovir and foscarnet are DNA polymerase-binding nucleotide analogs or pyrophosphate compounds, respectively. In contrast to interfering with viral DNA polymerase function, fomivirsen (formerly known as ISIS 2922) blocks viral replication by inhibiting translation of the essential HCMV immediate early protein IE2 (Azad et al., 1993; Lischka and Zimmermann, 2008). However, clinical use of the approved anti-HCMV compounds is restricted due to emergence of drug resistance, along with high cytotoxicity which can lead to severe adverse effects including leukopenia, thrombocytopenia, anemia, bone marrow cytotoxicity, nephrotoxicity as well as electrolyte abnormalities (Schreiber et al., 2009). During recent years, new anti-HCMV drugs were discovered and reached clinical development. Amongst them, artesunate, which is commonly used for treatment of severe malaria (Sinclair et al., 2009), has high potential to enrich the list of practicable treatments of HCMV infection in the future. Besides being safe and lacking severe adverse effects, artesunate inhibits a number of viruses in vitro, such as HCMV and other human herpesviruses, as well as hepatitis B and C virus (Milbradt et al., 2009a; Shapira et al., 2008; Kaptein et al., 2006; Efferth et al., 2002, 2008). To date, data suggest that artesunate does not directly attack a viral target. Artesunate downmodulates virus-supporting regulatory processes, like activation of NF-κB or Sp1 pathways and therefore interferes with critical host-cell and metabolism requirements for viral replication.
This cell-based targeting of the drug, interestingly, might reduce the risk of drug resistance development (Efferth et al., 2008). In order to further study the antiviral potential of artesunate, an initial clinical trial in stem cell transplant recipients receiving preemptive artesunate therapy has been performed (http://clinicaltrials.gov/ct2/show/NCT00284687; Shapira et al., 2008; Wolf et al., submitted to Clinical Infectious Diseases).

B-2 Replication cycle

Detailed knowledge of intracellular events during HCMV replication is not only crucial for identifying novel targets for antiviral therapy, but also for an improved understanding of fundamental principles of virus-host interaction. As a first step of HCMV infection, viral particles attach to the cell surface by low-affinity binding of glycoprotein gB to heparan sulfate proteoglycans (Revello and Gerna, 2010; Compton et al., 1993). Further interaction of the heteromeric glycoprotein complex gH-gL-gO with as yet poorly characterized or unidentified receptors is believed to be necessary for high-affinity binding and the subsequent fusion of the viral envelope with the cell membrane (Fig. 1, step 1) (Theiler and Compton, 2001). Candidates for cellular entry receptors have been suggested with integrins (Compton T., 2004), EGFR (Wang et al., 2003) and PDGFR (Soroceanu et al., 2008). After fusion, capsid-tegument complexes are released, together with the tegument, to the cytoplasm of infected cells (Fig. 1, step 2). Like most DNA viruses, HCMV replication comprises a nuclear phase. Consequently, the capsids are translocated through the cytoplasm to the nuclear proximity (Fig. 1, step 3) which is facilitated by interaction with cytoplasmic microtubules. This step is followed by docking of capsids to nuclear pores and the release of the viral DNA genome into the nucleus (Fig. 1, step 4) (Ogawa-Goto et al., 2003). Once the viral genome has reached the nucleus, the expression of viral ORFs is initiated. Gene expression during lytic HCMV replication occurs in a temporally regulated cascade consisting of three distinct phases, designated as immediate early (IE), early (E) and late (L) (Pellett and Roizman, 2007). As an initial event, viral tegument proteins directly trigger the initiation of IE protein expression. The two most abundant IE proteins, IE1p72 and IE2p86, are required for the efficient onset of the E phase due to their properties as transactivators of E genes and further regulatory functions. E genes encode numerous enzymes necessary for genome replication as well as transactivators of L gene expression. Basically, L proteins inherit functions in capsid assembly, DNA encapsidation, virion maturation and release of mature virions (Mocarski et al., 2007). Synthesis and accumulation of viral DNA occurs in the E phase in distinct nuclear regions termed replication compartments. HCMV DNA replication is based on a circularization of the linear genome and a process of concatamer formation for which a rolling circle model was described (Fig. 1, step 5) (Pari G. S., 2008). The concatameric DNA is cleaved by the viral terminase complex to produce genome-
length segments of viral DNA which are then inserted into the preformed capsids (Fig. 1, step 6 and 7) (Bogner E., 2002). Following encapsidation and capsid movement to the inner nuclear membrane (INM), primary tegumentation and primary envelopment of capsids are detectable. For this, a complex multi-stage envelopment-deenvelopment-reenvelopment process has been suggested (Lee and Chen, 2010; Mettenleiter et al., 2009). First, capsids obtain their primary envelope by budding at the INM (Fig. 1, step 8). The particles subsequently lose their primary envelope during translocation to the cytoplasm by fusion with the outer nuclear membrane (ONM) (Fig. 1, step 9). Whereas some tegument proteins already associate with viral capsids while budding through the INM, the final tegument is added in the cytoplasm (Fig. 1, step 10). Capsids are then transported via cytoplasmic microtubules to sites of secondary envelopment at vesicles of the trans-Golgi network (Mocarski et al., 2007; AuCoin et al., 2006). During budding into Golgi-derived vesicles, the capsids gain their final envelope with membrane-inserted viral glycoproteins (Fig. 1, step 11). Noteworthy, the endoplasmic reticulum (ER)-Golgi intermediate compartment (Sanchez et al., 2000) as well as endosomes (Das et al., 2007; Turcotte et al., 2005) have been suggested as potential further sites for secondary envelopment of HCMV and other herpesviruses. Mature viral particles, trapped in vesicles, are finally transported to the plasma membrane (Fig. 1, step 12) for terminal membrane fusion and release of infectious virions into the extracellular space (Fig. 1, step 13) (Mocarski et al., 2007).

**FIGURE 1. Intracellular transport pathways during HCMV replication.** This model represents schematically the current view of HCMV replication cycle, and was constructed according to previously published studies (Lee and Chen, 2010; Mettenleiter et al., 2009; Mocarski et al., 2007; Sanchez and Spector, 2002). See text for detailed description of individual steps. ER, endoplasmic reticulum.
Introduction

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B-3 Nuclear lamina as a physical barrier for HCMV nuclear egress

Translocation of viral capsids from the nucleus to the cytoplasm (nuclear egress) is one of the most crucial steps during replication of HCMV and other herpesviruses. The nuclear envelope consists of three distinct elements: nuclear membrane, nuclear pores and nuclear lamina. The nuclear membrane is composed of two phospholipid bilayers forming the ONM and INM. Together, the ONM, which is continuous with the ER, and the INM enclose a lumen of 25-40 nm thickness, termed perinuclear space (Margalit et al., 2005). The ONM and INM converge at nuclear pores, which are constituted by nuclear pore complexes (NPCs). Nuclear pores are embedded into the nuclear membrane and regulate the bidirectional transport of macromolecules between the cytoplasm and the nucleus (Cook et al., 2007). Due to their large size (~130 nm; Mocarski et al., 2007), HCMV capsids cannot be transported through nuclear pores (~40 nm; Panté and Kann, 2002). The enlargement of nuclear pores was occasionally observed in herpes simplex virus type 1 (HSV-1)-infected cells (Leuzinger et al., 2005), but was not conclusively demonstrated in HCMV-infected fibroblasts. Importantly, site-specific budding of viral capsids through distinct locally occurring invaginations in the INM of HCMV-infected cells could be illustrated by Buser et al. (2007) using electron microscopic analysis. Thus, it was suggested that herpesviral nuclear capsid egress through enlarged nuclear pores may be an auxiliary pathway subordinated to the proposed budding of capsids at the INM (Lee and Chen, 2010). However, access of herpesviral capsids to the INM is impeded by the proteinaceous network of the nuclear lamina. Lamins, belonging to type V intermediate filament proteins, are the main constituents of the nuclear lamina and are classified as A and B types. A-type lamins (A, C, AΔ10, and C2; collectively lamin A/C) result from alternative splicing of the LMNA gene. B-type lamins (collectively lamin B) are encoded by the LMNB1 (lamin B1) or LMNB2 (lamin B2 and lamin B3) gene (Gruenbaum et al., 2005; Goldman et al., 2002). In general, lamins are composed of a central α-helical coiled-coil rod domain flanked by globular head (N-terminal) and tail (C-terminal) domains. The rod domain mediates lamin dimerization, whereas the head and tail domains assemble to head-to-tail polymers forming an irregular filamentous meshwork (Dechat et al., 2008; Gruenbaum et al., 2005). Lamin A/C and lamin B differ in their ability to remain associated with the INM. Whereas lamin A/C can be found solubilized in the nucleus, lamin B is permanently membrane-associated due to post-translational isoprenylation and specific protein interactions with membrane proteins such as the lamin B receptor (LBR) (Goldberg et al., 2008). Due to interaction of lamins with lamina-associated membrane proteins, the nuclear lamina inherits roles in epigenetics, chromatin organization, DNA replication, transcription and DNA repair (Schirmer and Foisner, 2007; Vlcek and Foisner, 2007). For instance, the LBR, a polytopic protein with eight transmembrane domains interacting with lamin B, anchors the nuclear lamina and also heterochromatin to the nuclear membrane, thereby
influencing the organization and regulation of chromatin in the interphase nucleus (Wilson and Foisner, 2010; Worman et al., 1990). Furthermore, LBR possesses sterol reductase activity which might indicate a possible receptor function for sterol signaling-like molecules (Silve et al., 1998; Zwerger et al., 2010). A major function of the nuclear lamina is to maintain the structure of the nuclear environment (Dechat et al., 2008). Consequently, the nuclear lamina has to be transiently disassembled during mitotic breakdown of the nuclear envelope. Such mitosis-inherent processes appear to be subject to a reprogramming by the activity of viral nuclear egress proteins in HCMV-infected cells.

B-4 Phosphorylation-mediated disassembly of the nuclear lamina

The dynamic process of nuclear lamina disassembly is regulated by destabilizing phosphorylation of lamins at specific sites. In particular, it is well established that cyclin-dependent kinase 1 (CDK1; cdc2) is mainly responsible for phosphorylation of lamins during mitosis (Goldman et al., 2002; Ward and Kirchner, 1990; Peter et al., 1990). CDK1-dependent phosphorylation of lamin A/C occurs on serines at residues 22 (Ser22) and 392 (Ser392) with the probability of further unidentified sites (Hamirally et al., 2009; Heald and McKeon, 1990). In addition to CDK1, protein kinase C (PKC) is also important for lamin phosphorylation resulting in the nuclear lamina disassembly during apoptosis and mitosis (Cross et al., 2000; Thompson and Fields, 1996). Since the nuclear lamina provides a major obstacle for viral capsids to reach the INM, a similar destabilization of the nuclear lamina was suggested during replication of HCMV and other herpesviruses (Muranyi et al., 2002; Sanchez and Spector, 2002). Noteworthy, HCMV blocks the cell cycle through the action of viral regulatory proteins (Maul and Negorev, 2008; Bain and Sinclair, 2007). Thus, it remains unclear whether HCMV is able to utilize the CDK1-based pathway for distortion of the nuclear lamina. However, in cells infected with HSV-1 or murine CMV (MCMV), PKC is recruited to the nuclear envelope and is believed to be important for lamin phosphorylation and dissolution of the nuclear lamina (Park and Baines, 2006; Muranyi et al., 2002). Whether PKC or other cellular protein kinases are involved in the nuclear egress of HCMV has not been investigated so far. Particularly noteworthy, also herpesviruses encode protein kinases capable of phosphorylating nuclear lamins. α-herpesviruses (e.g. HSV-1, Pseudorabies virus; PrV) encode both US3 and UL13 protein kinases, while β-herpesviruses (e.g. HCMV, MCMV) and γ-herpesviruses (e.g. Epstein-Barr virus; EBV) only code for UL13 homologs (Gershburg and Pagano, 2008). α-herpesviral US3 is a serine/threonine (Ser/Thr) kinase which phosphorylates lamin A/C in vitro and is required for physiological lamin phosphorylation during viral replication. Since viral capsids accumulate in the perinuclear space in cells infected with US3-deletion virus, US3 was suggested to play a crucial role in the nuclear capsid egress of α-herpesviruses (Mou et al., 2007). UL13, belonging to the
so-called conserved herpesviral protein kinases, recognizes serine/threonine-proline (Ser/Thr-Pro) motifs in their substrates (Gershburg and Pagano, 2008; Lee and Chen, 2010). All UL13 homologs autophosphorylate and share functions that mimic cellular CDK activity, such as phosphorylation of the cellular translation factor EF-1α at the same site as CDK1 (Kawaguchi et al., 2003). Moreover, lamin phosphorylation mediated by UL13 homologs has also been described to be crucial for nuclear lamina disassembly during infection and critical for nuclear egress of all members of the Herpesviridae (Lee and Chen, 2010). In addition to the contribution of viral and cellular protein kinases, two conserved herpesvirus-encoded proteins were proven to be essential for budding of capsids at the INM (Mettenleiter et al., 2009). In particular, the well-studied HSV-1-encoded proteins UL34 and UL31 have been described as essential factors for primary envelopment (Reynolds et al., 2004). UL34 belongs to type II transmembrane proteins present in the nuclear membrane and the primary envelope of virions, while UL31 has been identified as a nuclear matrix-associated phosphoprotein (Mettenleiter et al., 2009; Reynolds et al., 2001). Together, UL34 and UL31 form complexes at the nuclear envelope and both are codependent for their proper subcellular localization at the nuclear rim (Reynolds et al., 2001). Functionally, UL34 and UL31 have a destabilizing effect on the structure of the nuclear lamina as a result of a direct interaction of UL34 and UL31 with lamin A/C. Importantly, further factors than these two conserved proteins appear to be required for efficient dissolution of the nuclear lamina (Reynolds et al., 2004; Bjerke and Roller, 2006). Hence, recent studies revealed that nuclear capsid egress of α-herpesviruses is dependent on the interplay of the UL34-UL31 complex with viral and cellular protein kinases (Lee and Chen, 2010). Viral US3 and UL13 are believed to induce the initial disassembly of the nuclear lamina during infection and further regulate the formation and proper localization of UL34 and UL31 by phosphorylation (Mou et al., 2009; Mou et al., 2007; Reynolds et al., 2002; Kato et al., 2006; Reynolds et al., 2001; Ryckman and Roller, 2004). As a next step, the UL34-UL31 complex also recruits cellular PKC to the nuclear lamina resulting in increased phosphorylation of lamins (Park and Baines, 2006). Ultimately, lamin phosphorylation results in a disassembly of the tight meshwork of the nuclear lamina in a manner that α-herpesviral capsids can attach to the INM to proceed budding into the perinuclear space (Baines et al., 2007). After primary envelopment, viral particles undergo deenvelopment by fusion with the ONM. For this step, involvement of viral glycoproteins and US3 kinase were suggested (Wisner et al., 2009; Farnsworth et al., 2007). Compared to α-herpesviruses, processes of nuclear capsid egress of β- and γ-herpesviruses appear somewhat different. Since US3 is unique for α-herpesviruses, kinase functions have to be adopted by the UL13 homologs or by cellular protein kinases. For HCMV, it has been demonstrated that pharmacological inhibition of pUL97 (HCMV-encoded homolog of UL13) specifically decreased cytoplasmic export of capsids, indicating that pUL97 is required at the stage of nuclear egress (Krosky et al., 2003). Furthermore, it has yet been demonstrated that
pUL97 phosphorylates lamins and has a destabilizing effect on the integrity of the nuclear lamina (Marschall et al., 2005). Interestingly, pUL97 is recruited to the LBR by interaction with the cellular protein p32. Originally identified as an interaction partner of splicing factor ASF/SF2, p32 regulates the splicing activity of ASF/SF2 by its own phosphorylation status (Kato et al., 2008). However, further biological functions of p32 and contribution to the efficiency of viral replication have been suggested. Particularly noteworthy, Muranyi et al. (2002) could illustrate that cellular PKC is recruited for phosphorylation and dissolution of the nuclear lamina during MCMV replication. In accordance to this, contribution of PKC in the nuclear egress of HCMV capsids seemed to be probable due to the fact that PKC activity is a known determinant of HCMV replication (Fortunato et al., 2000). Notably, in EBV, representative for γ-herpesviruses, the UL13 homolog BGLF4 appears to be sufficient to regulate the phosphorylation-dependent nuclear lamina disassembly during nuclear egress (Lee et al., 2008; Gershburg and Pagano, 2008). Further involvement of cellular protein kinases, however, could not be excluded (Lee and Chen, 2010). In addition to protein kinases, the homologs of HSV-1-encoded UL34 and UL31 of β-herpesviruses (HCMV, pUL50 and pUL53; MCMV, pM50 and pM53) and γ-herpesviruses (EBV, BFRF1 and BFLF2) are also crucial for nuclear capsid egress (Camozzi et al., 2008; Muranyi et al., 2002, Bubeck et al., 2004; Lötzerich et al., 2006; Rupp et al., 2007; Popa et al., 2010; Lake and Hutt-Fletcher, 2004; Granato et al., 2008). However, relatively little information was available for HCMV pUL50 and pUL53. Dal Monte et al. (2002) described a lamina association of pUL53 in HCMV-infected fibroblasts. In detail, pUL53 colocalized with lamin B and was incorporated into the virion tegument (Dal Monte et al., 2002). Furthermore, the direct interaction of pUL50 and pUL53 was illustrated (Milbradt et al., 2007) indicating that these two HCMV-encoded proteins behave similarly to their HSV-1 homologs. As an important feature, it could be demonstrated that the pUL50-pUL53 complex (with pUL50 as the essential determinant) efficiently recruits PKC to the nuclear envelope (Milbradt et al., 2007). Consequently, these results suggested that pUL50, and also pUL53 play an important regulatory role in nuclear egress. Thus, a hypothetical model is presented here illustrating potential processes during the nuclear egress of HCMV (Fig. 2). The phosphorylation-dependent dissolution of the nuclear lamina seems to be necessary for the nuclear egress of HCMV capsids. Mainly on the basis of observations of homologs in other herpesviruses, complex formation of pUL50 and pUL53 at the INM might be crucial for the recruitment of protein kinases (PKs). The lamin-phosphorylating pUL97 appears to be the most obvious candidate for this, though further contribution of cellular protein kinases appears probable. After the dissolution of the nuclear lamina, viral capsids gain access to the INM. At the INM, capsids can bud into the perinuclear space, undergoing primary envelopment. Finally, capsids proceed to egress into the cytoplasm by further budding through the ONM (Fig. 2).
FIGURE 2. Hypothetical model of HCMV nuclear capsid egress. Involvement of HCMV-encoded pUL50 and pUL53 in the nuclear egress of HCMV was deduced from studies of their homologous proteins in other herpesviruses (e.g. Muranyi et al., 2002; Reynolds et al., 2001; Fuchs et al., 2002; Farina et al., 2005). INM/ONM, inner/outer nuclear membrane; NPC, nuclear pore complex; PK, protein kinase (modified from Sanchez and Spector, 2002).
C Objectives

The nuclear egress of viral capsids is a rate-limiting step in the replication of the human cytomegalovirus (HCMV). The molecular events responsible for the translocation of viral capsids from the nucleus into the cytoplasm have not yet been fully understood. To date, the transient destabilization of the nuclear lamina is believed to be a prerequisite for the budding of viral capsids through the nuclear membrane. In particular, the disassembly of the nuclear lamina is achieved by the interplay of two lamina-associated viral proteins with viral and cellular lamin-phosphorylating protein kinases. Previously, the knowledge of processes required for nuclear egress of HCMV has mostly been derived from studies concerning other herpesviruses.

The aim of this study was to reveal the molecular basis of HCMV nuclear capsid egress, including the functional characterization of the nuclear egress proteins pUL50 and pUL53, and the identification of further proteins involved. First, yeast two-hybrid and coimmunoprecipitation analyses should be performed to determine potential viral and cellular interaction partners of pUL50 and/or pUL53. Since disassembly of the nuclear lamina is mediated by phosphorylation of lamins, reasonable candidates for interaction were protein kinases, such as viral pUL97 and cellular protein kinase C. Investigation of the subcellular localization of pUL50, pUL53 and possibly detected interaction partners by immunofluorescence analysis should elucidate the contribution of these proteins to the nuclear egress of HCMV. Second, the ability of protein kinases to reorganize the nuclear lamina should be analyzed in transient transfection experiments and in comparative settings with HCMV-infected fibroblasts. In addition, the use of specific protein kinase inhibitors and infection experiments with a recombinant UL97-deleted HCMV should clarify whether pUL97 and protein kinase C are involved in nuclear lamina destabilization during HCMV replication. Furthermore, disassembly of the nuclear lamina and the subsequent nuclear egress of viral capsids should be illustrated by high-resolution confocal microscopy and live-cell imaging using recombinant HCMV TB40 UL32-EGFP which produced GFP-labeled viral particles. Finally, emphasis should be put on the discovery of the molecular mechanism behind the nuclear lamina destabilization including involvement of potential further effector proteins. Together, these studies were thought to improve the understanding of molecular events required for the nuclear capsid egress of HCMV.
D  Material and Methods

D-1  Biological materials

D-1.1  Bacteria

*Escherichia coli* DH10B: F⁻ araD139 Δ(ara, leu) 7697 ΔlacX74 galU galK rpsL deoR
Φ80lacZΔM15 endA1 nupG recA1 mcrA Δ(mrr hsdRMS mcrBC) (Grant *et al.*, 1990)

D-1.2  Human cultured cells

**HEK293T**: human embryonic kidney epithelial cell line transformed by adenovirus type 5 that contains a genomic integrate encoding the simian virus 40 (SV40) large tumor antigen (Pear *et al.*, 1993)

**HeLa**: human cervical carcinoma cell line, positive for human papillomavirus type 16 (Nelson-Rees and Flandermeyer, 1976)

**HFF**: primary human foreskin fibroblasts

**U373-MG**: human glioblastoma cell line which is permissive for HCMV (Beckman *et al.*, 1971)

D-1.3  Yeast

**Saccharomyces cerevisiae** Y153: MATa leu2-3,112 ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δgal80ΔURA3::GAL-lacZ, LYS2::GAL-HIS3 (Durfee *et al.*, 1993)

D-1.4  Virus strains

**HCMV AD169**: laboratory strain of human cytomegalovirus (HCMV) originally isolated by Rowe and colleagues (Rowe *et al.*, 1956)

**BAC213 (HCMV AD169ΔUL97-GFP)**: UL97-deleted HCMV derived from AD169, containing an expression cassette for the green fluorescent protein (GFP) within nonessential stretches of the US region (Marschall *et al.*, 2005)

**HCMV TB40 UL32-EGFP**: recombinant virus derived from the endothelial cell-adapted HCMV strain TB40 coding for the capsid-associated tegument protein pUL32 (pp150) in fusion with enhanced GFP (Sampaio *et al.*, 2005)
D-1.5 Antibodies

D-1.5.1 Monoclonal antibodies

**mAb-FLAG (M2):** mouse monoclonal antibody directed against the DYKDDDDK peptide (FLAG epitope; Sigma-Aldrich, Deisenhofen, Germany)

**mAb-GFP (7.1/13.1):** mixture of two mouse monoclonal antibodies (clones 7.1 and 13.1) directed against GFP (Roche, Mannheim, Germany)

**mAb-HA (12CA5):** mouse monoclonal antibody directed against hemagglutinin (HA epitope; Roche, Mannheim, Germany)

**mAb-MYC (1-9E10.2):** mouse monoclonal antibody directed against the EQKLISEEDL peptide (MYC epitope; ATCC-LGC Standards GmbH, Wesel, Germany)

**mAb-β-actin (AC-15):** mouse monoclonal antibody for the detection of β-actin of human origin (Sigma-Aldrich, Deisenhofen, Germany)

**mAb-lamin A/C (636):** mouse monoclonal antibody for the detection of lamin A and lamin C of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

**mAb-LBR (E398L):** rabbit monoclonal antibody for the detection of the lamin B receptor (LBR) of human origin (Biomol GmbH, Hamburg, Germany)

**mAb-PKCα (A-3):** mouse monoclonal antibody raised against amino acids 373-672 of PKCα of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

**mAb-414:** mouse monoclonal antibody for the detection of the nuclear pore complex (NPC) proteins NUP62/152/90 (Hiss Diagnostics, Freiburg, Germany)

**mAb-UL44 (BS 510):** mouse monoclonal antibody for the detection of pUL44 of HCMV (kindly provided by Prof. B. Plachter, Mainz, Germany)

D-1.5.2 Polyclonal antibodies

**pAb-FLAG (F7425):** rabbit polyclonal antibody directed against the DYKDDDDK peptide (FLAG epitope; Sigma-Aldrich, Deisenhofen, Germany)

**pAb-HA (HA.11):** rabbit polyclonal antibody directed against hemagglutinin (HA epitope; Eurogentec Deutschland GmbH, Köln, Germany)

**pAb-calreticulin (PA3-900):** polyclonal antibody directed against calreticulin of human origin (Thermo Fisher Scientific, Bonn, Germany)

**pAb-Pin1 (H-123):** rabbit polyclonal antibody raised against amino acids 41-163 of Pin1 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); reactivity in indirect immunofluorescence assays

**pAb-Pin1 (A302-315A):** rabbit polyclonal antibody raised against amino acids 40-100 of Pin1 of human origin (Biomol GmbH, Hamburg, Germany); reactivity in coimmunoprecipitation assays
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**pAb-Pin1 (A302-316A):** rabbit polyclonal antibody raised against amino acids 113-163 of Pin1 of human origin (Biomol GmbH, Hamburg, Germany); reactivity in Western blot immunostainings

**pAb-p32:** rabbit polyclonal antibody raised against the C-terminus of p32 of human origin (kindly provided by Prof. W. C. Russel, St Andrews, UK)

**pAb-UL53:** mouse polyclonal antibody for the detection of pUL53 of HCMV (kindly provided by Dr. P. Dal Monte, Bologna, Italy)

**pAb-UL97:** rabbit polyclonal antibody for the detection of pUL97 of HCMV (kindly provided by Prof. D. Michels, Ulm, Germany)

**preimmune serum (pre):** rabbit polyclonal antibody received from blood samples taken prior to immunization (inventory of the Marschall laboratory)

**D-1.5.3 Secondary antibodies**

All secondary antibodies coupled to horseradish peroxidase (HRP) or conjugated with fluorescent dyes were purchased from Dianova (Hamburg, Germany).

- HRP-coupled goat anti-mouse IgG (H+L)
- HRP-coupled goat anti-rabbit IgG (H+L)
- Alexa 488-conjugated goat anti-mouse IgG (H+L)
- Alexa 488-conjugated goat anti-rabbit IgG (H+L)
- Alexa 555-conjugated goat anti-mouse IgG (H+L)
- Alexa 555-conjugated goat anti-rabbit IgG (H+L)

**D-2 Nucleic acids**

**D-2.1 Oligonucleotides**

All nucleotides were purchased from Biomers.net GmbH (Ulm, Germany). The sequences of oligonucleotides (annotated from 5´ to 3´) used for nucleotide sequencing, PCR cloning or PCR mutagenesis are listed below.

**Sequencing**

**UL50-for (nt554):**
GCAGTCACAGCGGCAGCG

**UL50-rev (nt687):**
GGTGGAGAGCAACTCGG

**UL53-for (nt503):**
CCACCAACCAGCCGCCC
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UL53-rev (nt648):
GCAGTCGCAGGGGATGTG

T7:
TTAATACGACTCACTATAGGG

Sp6:
GGGGCAAACAACAGATG

pGBT9-rev-1051:
GCAACCTGACCTACAGG

pGAD424-3’:
TGGTGCACGATGCACAGTTGAAGTG

Gal119:
TCT AAC ATT GAG ACA GCA TAG

Gal843:
GCG TTT GGA ATC ACT ACA GGG

PCR cloning

The sequence corresponding to the gene of interest is underlined. Restriction enzyme cleavage sites are highlighted in bold and the sequences for the FLAG or HA epitopes are in italics.

5’-PKCalpha-EcoRI:
TGAGAATTCATGGCTGACGTTTCCCGGGCAACG

5’-UL50-BamHI-EcoRI:
TGAGGATCCAGGAATTCATGGAGATGAACAGGTTCATCGCATC

5’-UL50(1)-HindIII:
TGAAAGCTTATGGAGATGAACAGGTTCATCGCATC

5’-UL50(1)-Xhol-C1-GFP:
TGACTCGAGATGGAGATGAACAGGTTCATCGCATC

5’-UL50(1)-Xhol-N1-GFP:
TGACTCGAGATGGAGATGAACAGGTTCATCGCATC

5’-UL50(5)-EcoRI:
TGAGAATTCATGAGGTTTCATCGGATCTGGTGAGGCC
5-UL50(10)-EcoRI:
TGAGAATTCATGGATCTGGTGCAAGCCACCGGCCTATCC

5-UL50(15)-EcoRI:
TGAGAATTCATGACCGGGCTATCCTCAAGTTGGGTCCC

5-UL50(20)-BamHI-EcoRI:
TGAGGATCCAGGAATTCGATGAAGTTGGGTCAccAGCGAGCTGCGCG

5-UL50(40)-BamHI-EcoRI:
TGAGGATCCAGGAATTCGATGTAATCGGTGTGCGACGCGCCATGCTCA

5-UL50(70)-BamHI-EcoRI:
TGAGGATCCAGGAATTCGATGCGCTTTGTTTATCTTTTTAAAAACACTG

5-UL50(100)-BamHI-EcoRI:
TGAGGATCCAGGAATTCGATGGTAGGTGAGTTCATGTGCTTAAGG

5-UL50(150)-BamHI-EcoRI:
TGAGGATCCAGGAATTCGATGGCCTTTCCGACCCCAGAAAAACGAGGGCG

5-UL50(250)-XhoI-C1-GFP:
TGACTCGAGGAATGGGGTCGTGCGCGGCTGTGCTG

5-UL50(250)-XhoI-N1-GFP:
TGACTCGAGGAATGGGGTCGTGCGCGGCTGTGCTG

5-UL50(250)-XhoI-C1-GFP:
TGACTCGAGGAATGGGGTCGTGCGCGGCTGTGCTG

5-UL50(250)-XhoI-N1-GFP:
TGACTCGAGGAATGGGGTCGTGCGCGGCTGTGCTG

3-PKCalpha(672)-FLAG-XhoI:
TGACTCGAGTCACTTGTGCATCCTCGTTTTGTAGTCTACTGCACTCTGTAAGATGGGGGTGC

3-PKCalpha(597)-FLAG-XhoI:
TGACTCGAGTCACTTGTGCATCCTCGTTTTGTAGTCGAAGAAGGCATGCTCTCTCACGTCC

3-PKCalpha(510)-FLAG-XhoI:
TGACTCGAGTCACTTGTGCATCCTCGTTTTGTAGTCGATTATCTCTCGGGGCGATATAATCT

3-PKCalpha(424)-FLAG-XhoI:
TGACTCGAGTCACTTGTGCATCCTCGTTTTGTAGTCGGCCCACCACGTGACATATGCCATG
3-PKCalpha(339)-FLAG-Xhol:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(397)-HA-XholPst:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(358)-EcoRI-C1-GFP:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(358)-EcoRI-N1-GFP:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(340)-HA-XholPst:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(310)-HA-XholPst:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(280)-EcoRI-C1-GFP:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(280)-EcoRI-N1-GFP:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(250)-HA-XholPst:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(280)-HA-XholPst:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(181)-BamHI:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(150)-BamHI:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA

3-UL50(130)-BamHI:
TGAACGCATGCTGTCATCTGGCTCTTGAGTCTGAAGTCCGTGAGTTTCACTCGGTCA
3-UL50(100)-BamHI: 
TGAGGATCCGGTGACGTGGCGCCCGCAGCAAACGAAGCTTGAC

3-UL50(70)-BamHI: 
TGAGGATCCGGAGGACGTGGCTGTGCGGCTCTCCAGTA

3-UL50(20)-EcoRI-C1-GFP: 
TGAGAATTCTACTGGAGATACGCCGCGTGGCCT

3-UL50(20)-EcoRI-N1-GFP: 
TGAGAATTCTCTGAGATACGCCGCGTGGCCT

PCR mutagenesis

The sequence corresponding to the gene of interest is underlined. Nucleotides differing from the wild-type sequence are shaded in grey.

5-UL50(D10A): 
GGTTCTCCATCAGGCACTGGTGACGGCCACGCGGC

3-UL50(D10A): 
CGCCCGTGGCCCTGACCAGTGGCCTGAGAACC

5-UL50(L11A/V12A): 
GGTTCTCCATCAGGCACTGGTGACGGCCACGCGGC

3-UL50(L11A/V12A): 
CGCCCGTGGCCCTGACCAGTGGCCTGAGAACC

5-UL50(Q13A): 
GGTTCTCCATCAGGCACTGGTGACGGCCACGCGGC

3-UL50(Q13A): 
CGCCCGTGGCCCTGACCAGTGGCCTGAGAACC

D-2.2 Vectors and expression plasmids

D-2.2.1 Eukaryotic cloning vectors

pcDNA3.1: mammalian expression vector containing a multiple cloning site (MCS) for insertion of an open reading frame of interest under the control of the HCMV immediate/early promoter/enhancer; for selection of transfected cell clones, the vector carries neomycin and ampicillin resistance cassettes (Invitrogen, Karlsruhe, Germany)
**pDS-Red1-N1**: mammalian expression vector encoding the red fluorescent protein (RFP); genes cloned into the MCS are expressed as fusions to the N-terminus of RFP; for selection of transfected cell clones, the vector carries the neomycin/kanamycin resistance cassette (Clontech, Palo Alto, CA, USA)

**pEGFP-C1**: mammalian expression vector encoding the enhanced green fluorescent protein (eGFP); genes cloned into the MCS are expressed as fusions to the C-terminus of eGFP; for selection of transfected cell clones, the vector carries the neomycin/kanamycin resistance cassette (Clontech, Palo Alto, CA, USA)

**pEGFP-N1**: mammalian expression vector encoding eGFP; genes cloned into the MCS are expressed as fusions to the N-terminus of eGFP; for selection of transfected cell clones, the vector carries the neomycin/kanamycin resistance cassette (Clontech, Palo Alto, CA, USA).

**pGAD424**: yeast expression vector containing the GAL4 activation domain (AD) under the control of the ADH promoter and the leucine marker (LEU2 gene) for selection in yeast (BD Biosciences Clontech, Mountain View, CA, USA)

**pGBT9**: yeast expression vector containing the GAL4 DNA binding domain (BD) under the control of the ADH promoter and the tryptophane marker (TRP1 gene) for selection in yeast (BD Biosciences Clontech, Mountain View, CA, USA)

### D-2.2.2 Ready-to-use plasmids

**Mammalian expression plasmids**

**pcDNA-UL13-HA (pHM2395)**: eukaryotic expression plasmid encoding HSV-1 UL13, C-terminally fused to the HA epitope (inventory of the Marschall laboratory)

**pcDNA-F-UL26 (pHM1808)**: eukaryotic expression plasmid encoding amino acids 1-223 of pUL26, N-terminally fused to the FLAG epitope (Lorz et al., 2006; Marschall et al., 2003)

**pcDNA-UL50-F (pHM2588)**: eukaryotic expression plasmid encoding pUL50, C-terminally fused to the FLAG epitope (Milbradt et al., 2007)

**pcDNA-UL50-HA (pHM2589)**: eukaryotic expression plasmid encoding pUL50, C-terminally fused to the HA epitope (Milbradt et al., 2007)

**pcDNA-UL50(1-358)-HA (pHM2577)**: eukaryotic expression plasmid encoding amino acids 1-358 of pUL50, C-terminally fused to the HA epitope (Milbradt et al., 2007)

**pcDNA-UL53-F (pHM2590)**: eukaryotic expression plasmid encoding pUL53, C-terminally fused to the FLAG epitope (Milbradt et al., 2007)

**pcDNA-UL53-HA (pHM2591)**: eukaryotic expression plasmid encoding pUL53, C-terminally fused to the HA epitope (Milbradt et al., 2007)

**pcDNA-UL97-F (pF721)**: eukaryotic expression plasmid encoding pUL97, C-terminally fused to the FLAG epitope (Marschall et al., 2001)
**pcDNA-UL97-HA (pF722):** eukaryotic expression plasmid encoding pUL97, C-terminally fused to the HA epitope (Marschall et al., 2001)

**pcDNA-UL97(K355M)-F (pF715):** eukaryotic expression plasmid encoding pUL97 carrying an inactivating point mutation in the essential lysine codon of the kinase ATP binding site, C-terminally fused to the FLAG epitope (Marschall et al., 2001)

**pcDNA-UL97(1-595)-F (pHM1705):** eukaryotic expression plasmid encoding amino acids 1-595 of pUL97, C-terminally fused to the FLAG epitope (Marschall et al., 2005)

**pcDNA-UL97(181-707)-F (pHM1708):** eukaryotic expression plasmid encoding amino acids 181-707 of pUL97, C-terminally fused to the FLAG epitope (Marschall et al., 2005)

**pcDNA-UL97(231-707)-HA (pHM2433):** eukaryotic expression plasmid encoding amino acids 231-707 of pUL97, C-terminally fused to the HA epitope (Schregel et al., 2007)

**pcDNA5/FRT-p32-F (pHM2053):** eukaryotic expression plasmid encoding p32, C-terminally fused to the FLAG epitope (Marschall et al., 2005)

**pcDNA5/FRT-p32(50-282)-F (pHM2055):** eukaryotic expression plasmid encoding amino acids 50-282 of p32, C-terminally fused to the FLAG epitope (Marschall et al., 2005)

**peGFP-N1-IE2p86 (pHM990):** eukaryotic expression plasmid encoding IE2p86, C-terminally fused to eGFP (kindly provided by Dr. N. Tavalai, Erlangen, Germany; Kronschnabl et al., 2002)

**peGFP-N1-PKCα:** eukaryotic expression plasmid encoding PKCα, C-terminally fused to eGFP (kindly provided by PD Dr. G. Zimmer, Mittelhäusern, Switzerland)

**peGFP-N1-PKCγ:** eukaryotic expression plasmid encoding PKCγ, C-terminally fused to eGFP (kindly provided by PD Dr. G. Zimmer, Mittelhäusern, Switzerland)

**Yeast expression plasmids**

**pACT-p32 (pHM1486):** yeast expression plasmid encoding p32 fused to the GAL4 AD (Marschall et al., 2005)

**pACT-UL97 (pHM892):** yeast expression plasmid encoding pUL97 fused to the GAL4 AD (Marschall et al., 2003; Marschall et al., 2005)

**pADT7-PKCε:** yeast expression plasmid encoding PKCε fused to the GAL4 AD (kindly provided by Prof. T. Arimura, Tokyo, Japan; Arimura et al., 2004)

**pADT7-PKCζ:** yeast expression plasmid encoding PKCζ fused to the GAL4 AD (kindly provided by Prof. T. Arimura, Tokyo, Japan; Arimura et al., 2004)

**pAS-p32 (pHM1485):** yeast expression plasmid encoding p32 fused to the GAL4 BD (Marschall et al., 2005)

**pAS-UL97 (pHM870):** yeast expression plasmid encoding pUL97 fused to the GAL4 BD (Marschall et al., 2003; Marschall et al., 2005)

**pBKT7-PKCε:** yeast expression plasmid encoding PKCε fused to the GAL4 BD (kindly provided by Prof. T. Arimura, Tokyo, Japan; Arimura et al., 2004)
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**pBKT7-PKCζ:** yeast expression plasmid encoding PKCζ fused to the GAL4 BD (kindly provided by Prof. T. Arimura, Tokyo, Japan; Arimura et al., 2004)

**pGBT-LBR(1-208):** yeast expression plasmid encoding amino acids 1-208 of LBR fused to the GAL4 BD (kindly provided by Prof. H. J. Worman, Columbia University, NY, USA; Marschall et al., 2005)

**pGAD-UL50(1-358)-F (pHM2597):** yeast expression plasmid encoding amino acids 1-358 of pUL50, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 AD (Milbradt et al., 2007)

**pGAD-UL50(1-358/382-397)-F (pHM2598):** yeast expression plasmid encoding pUL50 with an internal deletion of amino acids 359-381, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 AD (Milbradt et al., 2007)

**pGAD-UL53-F (pHM2599):** yeast expression plasmid encoding pUL53, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 AD (Milbradt et al., 2007)

**pGBT-UL50(1-358)-F (pHM2593):** yeast expression plasmid encoding amino acids 1-358 of pUL50, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 BD (Milbradt et al., 2007)

**pGBT-UL50(1-358/382-397)-F (pHM2594):** yeast expression plasmid encoding pUL50 with an internal deletion of amino acids 359-381, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 BD (Milbradt et al., 2007)

**pGBT-UL53-F (pHM2595):** yeast expression plasmid encoding pUL53, C-terminally fused to the FLAG epitope and N-terminally fused to the GAL4 BD (Milbradt et al., 2007)

**pTD1:** yeast expression plasmid encoding the SV40 large T antigen fused to the GAL4 AD in pGAD3F with the leucine marker (LEU2 gene) for selection (Chien et al., 1991)

**pVA3:** yeast expression plasmid encoding the murine p53 protein fused to the GAL4 BD in pGBT9 with the tryptophane marker (TRP1 gene) for selection (Iwabuchi et al., 1993)

### 2.2.3 Newly generated plasmids

**Mammalian expression plasmids**

The following mammalian expression plasmids code for full-length and C-terminally truncated versions of PKCα, C-terminally fused to the FLAG epitope. The PKCα-FLAG fragments were generated by PCR amplification of the PKCα coding sequence using peGFP-N1-PKCα as template and oligonucleotide primers as indicated. Then the fragments were inserted into pcDNA3.1 via EcoRI and XhoI.
Material and Methods

The following mammalian expression plasmids code for truncated versions of pUL50 C-terminally fused to the HA epitope. The UL50-HA fragments were generated by PCR amplification of the UL50 coding sequence using pcDNA-UL50-HA (pHM2589) as template and oligonucleotide primers as indicated. Then the fragments were inserted into pcDNA3.1 via EcoRI and XhoI.

pcDNA-UL50(1-340)-HA (pHM2846): 5-UL50-BamHI-EcoRI, 3-UL50(340)-HA-XhoI-PstI
pcDNA-UL50(1-310)-HA (pHM3185): 5-UL50-BamHI-EcoRI, 3-UL50(310)-HA-XhoI-PstI
pcDNA-UL50(1-280)-HA (pHM2847): 5-UL50-BamHI-EcoRI, 3-UL50(280)-HA-XhoI-PstI
pcDNA-UL50(1-250)-HA (pHM2848): 5-UL50-BamHI-EcoRI, 3-UL50(250)-HA-XhoI-PstI
pcDNA-UL50(5-397)-HA (pHM3058): 5-UL50(5)-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(10-397)-HA (pHM3059): 5-UL50(10)-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(15-397)-HA (pHM3060): 5-UL50(15)-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(20-397)-HA (pHM2849): 5-UL50(20)-BamHI-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(40-397)-HA (pHM2850): 5-UL50(40)-BamHI-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(70-397)-HA (pHM3186): 5-UL50(70)-BamHI-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(100-397)-HA (pHM2851): 5-UL50(100)-BamHI-EcoRI, 3-UL50(397)-HA-XhoI-PstI
pcDNA-UL50(150-397)-HA (pHM2852): 5-UL50(150)-BamHI-EcoRI, 3-UL50(397)-HA-XhoI-PstI

The following mammalian expression plasmids code for truncated versions of pUL50 C-terminally fused to egFP. The UL50 fragments were generated by PCR amplification of the UL50 coding sequence using pcDNA-UL50-HA (pHM2589) as template and oligonucleotide primers as indicated. Then the fragments were inserted into peGFP-N1 via HindIII and BamHI.

peGFP-N1-UL50(1-181) (pHM3061): 5-UL50(1)-HindIII, 3-UL50(181)-BamHI
peGFP-N1-UL50(1-150) (pHM3062): 5-UL50(1)-HindIII, 3-UL50(150)-BamHI
peGFP-N1-UL50(1-130) (pHM3063): 5-UL50(1)-HindIII, 3-UL50(130)-BamHI
peGFP-N1-UL50(1-100) (pHM3064): 5-UL50(1)-HindIII, 3-UL50(100)-BamHI
peGFP-N1-UL50(1-70) (pHM3065): 5-UL50(1)-HindIII, 3-UL50(70)-BamHI

The following mammalian expression plasmids code for short amino acid stretches of pUL50 C- or N-terminally fused to egFP. The UL50 fragments were generated by PCR amplification of
the UL50 coding sequence using pcDNA-UL50-HA (pHM2589) as template and oligonucleotide primers as indicated. Then the fragments were inserted into peGFP-N1 or peGFP-C1 via Xhol and EcoRI.

\[
\begin{align*}
\text{peGFP-C1-UL50(1-20) (pHM2982):} & \quad 5\text{-UL50(1)}-\text{Xhol-C1-GFP}, \\
& \quad 3\text{-UL50(20)}-\text{EcoRI-C1-GFP} \\
\text{peGFP-C1-UL50(250-280) (pHM2983):} & \quad 5\text{-UL50(250)}-\text{Xhol-C1-GFP}, \\
& \quad 3\text{-UL50(280)}-\text{EcoRI-C1-GFP} \\
\text{peGFP-C1-UL50(340-358) (pHM2984):} & \quad 5\text{-UL50(340)}-\text{Xhol-C1-GFP}, \\
& \quad 3\text{-UL50(358)}-\text{EcoRI-C1-GFP} \\
\text{peGFP-N1-UL50(1-20) (pHM2979):} & \quad 5\text{-UL50(1)}-\text{Xhol-N1-GFP}, \\
& \quad 3\text{-UL50(20)}-\text{EcoRI-N1-GFP} \\
\text{peGFP-N1-UL50(250-280) (pHM2980):} & \quad 5\text{-UL50(250)}-\text{Xhol-N1-GFP}, \\
& \quad 3\text{-UL50(280)}-\text{EcoRI-N1-GFP} \\
\text{peGFP-N1-UL50(340-358) (pHM2981):} & \quad 5\text{-UL50(340)}-\text{Xhol-N1-GFP}, \\
& \quad 3\text{-UL50(358)}-\text{EcoRI-N1-GFP}
\end{align*}
\]

The following mammalian expression plasmids code for mutant pUL50, carrying single amino acid exchanges in the N-terminus, C-terminally fused to the HA epitope. The UL50-HA point mutants were generated by site-directed mutagenesis using pcDNA-UL50-HA (pHM2589) as template and oligonucleotide primers as indicated.

\[
\begin{align*}
\text{pcDNA-UL50(D10A) (pHM3294):} & \quad 5\text{-UL50(D10A)}, 3\text{-UL50(D10A)} \\
\text{pcDNA-UL50(L11A/V12A) (pHM3295):} & \quad 5\text{-UL50(L11A/V12A)}, 3\text{-UL50(L11A/V12A)} \\
\text{pcDNA-UL50(Q13A) (pHM3298):} & \quad 5\text{-UL50(Q13A)}, 3\text{-UL50(Q13A)}
\end{align*}
\]

D-2.3 Additional nucleic acids

GeneRuler™ 1kb DNA Ladder and GeneRuler™ Low Range DNA Ladder for determination of size and approximate yield of double-stranded DNA in agarose gels was obtained from Fermentas (St. Leon-Rot, Germany). Salmon testes DNA for yeast transformation was obtained from Sigma-Aldrich (Deisenhofen, Germany).

D-3 Enzymes, chemicals and media

D-3.1 Enzymes

The applied restriction endonucleases were purchased from Roche (Mannheim, Germany), Gibco/BRL (Eggenstein, Germany), New England Biolabs (Frankfurt am Main, Germany) or
Pharmacia (Freiburg, Germany) and used with provided buffers according to the manufacturers’ protocols. T4 DNA ligase (Gibco/BRL, Eggenstein, Germany), and VentR® DNA Polymerase (New England Biolabs, Frankfurt am Main, Germany) were utilized with buffers recommended by the manufacturers and according to the manufacturers’ protocols.

D-3.2 Media

D-3.2.1 Bacterial media

**LB medium (Luria-Bertani medium):** 10 g of bactotryptone, 5 g of bacto yeast, 8 g of NaCl and 1 g of glucose were dissolved in 1 liter of H₂O and adjusted to pH 7.2 using NaOH followed by autoclaving; optionally, ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added to the media.

**LB agar (Luria-Bertani agar):** 15 g of agar were dissolved in 1 liter of LB medium followed by autoclaving; after cooling of the solution to about 55°C, 1 ml of ampicillin (50 mg/ml) or kanamycin (15 mg/ml) was added.

**SOC medium:** 20 g of bactotryptone, 5 g of bacto yeast, 2.5 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose were dissolved in 1 liter of H₂O followed by filter sterilization.

D-3.2.2 Cell culture media

**MEM (Eagle’s minimal essential medium):** this medium was obtained from Gibco/BRL (Eggenstein, Germany) as a ready-to-use substance, dissolved in sterile H₂O and adjusted to a pH of 7.0.

**DMEM (Dulbecco’s modified Eagle medium):** this medium was obtained from Gibco/BRL (Eggenstein, Germany) as a ready-to-use substance, dissolved in sterile H₂O and adjusted to a pH of 7.0.

**FCS (fetal calf serum):** FCS was obtained from Cambrex (Verviers, Belgium).

**Trypsin/EDTA:** 0.25% trypsin, 140 mM NaCl, 5 mM KCl, 0.56 mM Na₂HPO₄, 5 mM D(+)-glucose, 25 mM Tris/HCl, 0.01% EDTA, pH 7.0.

D-3.2.3 Yeast media

**10x Yeast Nitrogen Base (YNB):** 33.5 g YNB lacking amino acids and ammonium sulfate were dissolved in 500 ml H₂O followed by sterile filtration.

**20x amino acid mix:** 0.9 g adenine, 0.43g arginine, 2.16 g aspartic acid, 2.16 g glutamic acid, 0.65 g isoleucine, 0.65 g lysine, 0.43 g methionine, 1.08 g phenylalanine, 7.92 g serine, 4.32 g threonine, 0.65 g tyrosine and 3.24 g valine were dissolved in 900 ml H₂O followed by sterile filtration.

**100x uracil:** 0.24 g uracil were dissolved in 100 ml H₂O followed by sterile filtration.
Material and Methods

200x histidine: 0.48 g histidine were dissolved in 100 ml H₂O followed by sterile filtration
200x leucine: 1.44 g leucine were dissolved in 100 ml H₂O followed by sterile filtration
200x tryptophane: 0.96 g tryptophane were dissolved in 100 ml H₂O followed by sterile filtration
YAPD: 10 g bacto yeast and 20 g bacto peptone were dissolved in 450 ml H₂O and autoclaved followed by addition of 50 ml 20% glucose

Minimal medium: 50 ml 10x YNB, 50 ml 20% glucose, 25 ml 20x amino acid mix, 5 ml 100x uracil and according to requirements 2.5 ml 200x histidine, 2.5 ml 200x tryptophane and 2.5 ml 200x leucine were dissolved in 500 ml H₂O

Minimal agar: 10 g bacto agar were dissolved in 300 ml H₂O and autoclaved. After cooling to about 55°C, supplements used for minimal medium were added according to requirements, and the final volume was adjusted to 500 ml; thereafter, the agar was casted into petri dishes

D-3.3 Chemicals

Chemicals for laboratory use were purchased from Biomol (Hamburg, Germany), Boehringer (Mannheim, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Deisenhofen, Germany).

D-3.4 Standard buffers and solutions

PBSO (phosphate-buffered saline without CaCl₂ and MgCl₂): 138 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄
6x DNA loading buffer: 30% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanole
1x TAE buffer: 24.2 g Tris base, 1.7 g EDTA, 5.7 ml glacial acetic acid were dissolved in H₂O adjusting the volume to 5 liters
1x TE buffer: 10 mM Tris/HCl (pH 6.8), 1 mM EDTA
4x SDS sample buffer: 125 mM Tris/HCl (pH 6.8), 2 mM EDTA, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.01% bromphenol blue
10x SDS-PAGE buffer: 286 g glycine, 60.6 g Tris base and 20 g SDS were dissolved in H₂O adjusting the volume to 2 liters
Western blotting buffer: 15.1 g Tris base, 75 g glycine and 1 liter ethanol were dissolved in H₂O adjusting the volume to 5 liters

ECL solution A: 50 mg luminol (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 200 ml 0.1 M Tris/HCl (pH 8.6)
ECL solution B: 11 mg p-hydroxycoumarin acid (Sigma-Aldrich, Deisenhofen, Germany) were dissolved in 10 ml DMSO
4% paraformaldehyde solution: 4 g paraformaldehyde was dissolved in 50 ml H₂O including some drops of a 1 N NaOH solution at 60°C and after cooling to room temperature (Rt) the solution was mixed with 50 ml 2x PBSo

0.2% Triton X-100: 0.2% Triton X-100 dissolved in PBSo

HBS solution (HEPES-buffered saline): 4.4 g NaCl and 2.4 g HEPES were dissolved in 500 ml H₂O and adjusted to pH 7.4 followed by sterile filtration

PEI2000 solution: 9 mg polyethyleneimine MW 2000 (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in 10 ml H₂O and adjusted to pH 7.0 followed by sterile filtration

PEI25000 solution: 9 mg polyethyleneimine MW 25000 (Sigma-Aldrich, Deisenhofen, Germany) was dissolved in 10 ml H₂O and adjusted to pH 7.0 followed by sterile filtration

RIPA lysis buffer: 0.1% SDS, 1% Na-desoxycholate, 10 mM Tris/Cl pH 7.5, 150 mM NaCl were dissolved in sterile H₂O followed by sterile filtration; shortly before usage 2 mM PMSF was added

Coimmunoprecipitation (CoIP) buffer: 25 ml 1 M Tris/HCl pH 8.0, 15 ml 5 M NaCl, 5 ml 0.5 M EDTA and 25 ml 10% NP40 were dissolved in H₂O adjusting volume to 500 ml followed by sterile filtration; shortly before usage 100 μl 100 mM PMSF, 20 μl 1 mg/ml aprotinin, 20 μl 1 mg/ml leupeptin and 20 μl 1 mg/ml pepstatin were added per 10 ml CoIP stock solution

PKC IVKA washing buffer: 20 mM HEPES, 0.03% Triton X-100 and 10 mM magnesium acetate were dissolved in H₂O and adjusted to pH 7.4 followed by sterile filtration

PKC IVKA buffer: 0.1 mg phosphatidylserine and 10 μg diacylglycerol were dissolved shortly before usage in 1 ml PKC IVKA washing buffer

Lithium acetate solution: 0.2 M lithium acetate dissolved in H₂O and adjusted to pH 8.5 followed by sterile filtration

50% polyethylenglycol (PEG): 50% PEG dissolved in H₂O followed by sterile filtration

Buffer Z: 16.1 g Na₃HPO₄ x 7H₂O, 5.8 g NaH₂PO₄ x H₂O, 0.75 g KCl and 0.246 g MgSO₄ x 7H₂O were dissolved in 1 liter H₂O and adjusted to pH 7.0 followed by autoclaving

Xgal: 20 mg Xgal were dissolved in 20 ml dimethylformamide

D-4 Protein kinase inhibitors

The indolocarbazoles staurosporine (STP; Sigma-Aldrich, Deisenhofen, Germany) and Gö6976 (Merck, Darmstadt, Germany) were used as inhibitors of PKC and the HCMV-encoded kinase pUL97. The tyrosine kinase inhibitor AG490 (tyrphostin; Sigma-Aldrich, Deisenhofen, Germany) served as control. All compounds were prepared in dimethyl sulfoxide (DMSO) and aliquots were stored at -20°C.
D-5 Standard molecular biology techniques

PCR (polymerase chain reaction) for amplification of DNA fragments according to Sambrook et al. (1989); DMSO, formamide or MgSO₄ were added to the PCR sample if it was necessary to improve the efficiency of the reaction

restriction enzyme digestion of DNA, ligation with T4 DNA ligase and agarose gel electrophoresis (Sambrook et al., 1989)

elution of DNA fragments from agarose gels by using commercial kits from either Invitrogen (Karlsruhe, Germany; PureLink™ Quick Gel Extraction Kit) or from Qiagen (Hilden, Germany; QIAquick Gel Extraction Kit)

transformation of plasmid DNA into bacteria by electroporation (Sambrook et al., 1989)

small-scale DNA preparation (Mini-preparation) by standard alkaline lysis procedure (Zagursky and Berman, 1984)

large-scale DNA preparation (Midiprep) by PureLink™ HiPure Plasmid Midiprep Kit obtained from Invitrogen (Karlsruhe, Germany)

photometric determination of DNA concentrations (Sambrook and Russel, 2001)

automated nucleotide sequencing of DNA using fluorescence-based ABI-Prism 2000 sequencing detector (ABI, Weiterstadt, Germany)

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Laemmli U. K., 1970)

enhanced chemiluminescence (ECL) immunodetection of proteins (Amersham, Braunschweig, Germany).

D-6 Cell culture techniques

D-6.1 Maintenance of cell cultures

Eukaryotic cell cultures were maintained in plastic flasks (Nunc, Roskilde, Denmark) at 37°C, 5% CO₂ and 80% humidity in corresponding culture media.

HEK293T: DMEM supplemented with 10% (v/v) FCS, 350 μg/ml l-glutamine and 10 μg/ml gentamicin

HeLa: DMEM supplemented with 10% (v/v) FCS, 350 μg/ml l-glutamine and 10 μg/ml gentamicin

HFF: MEM medium supplemented with 7.5% (v/v) FCS, 350 μg/ml l-glutamine, and 10 μg/ml gentamicin

U373-MG: DMEM supplemented with 10% (v/v) FCS, 350 μg/ml l-glutamine and 10 μg/ml gentamicin
After reaching a confluent monolayer, the adherent growing cells were detached from the surface by trypsin/EDTA treatment and reseeded into new flasks containing fresh medium.

D-6.2 Transfection of cultured cells

One day prior to transfection, 293T cells were seeded in 10 ml of medium into 10 cm dishes (5 x 10^6 cells per dish) or HeLa cells in 3 ml of medium into wells of a six-well plate on coverslips (3.2 x 10^5 cells per well). Plasmid transfection of 293T cells was performed by using the polyethyleneimine reagent (PEI; Schregel et al., 2007). For this, 12 μg of total DNA were mixed with 1 ml HBS solution and incubated with 20 μl PEI2000 in 1 ml HBS solution for 20 min at Rt. Thereafter, the solution was mixed with 36 μl PEI25000 in 1 ml HBS solution and incubated for 20 min at Rt. Within this time period, cell monolayers were washed twice with DMEM supplemented with l-glutamine and cultured in 4 ml of the medium in the absence of antibiotics. Then, the DNA-PEI mixture was added dropwise to the cells. After incubation for 3-4 h at 37°C, the transfection solution was replaced by fresh medium. At 48 h post-transfection, the cells were harvested for Western blot analysis, coimmunoprecipitation or in vitro kinase assay. Transfection of HeLa cells was performed by using the lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany). For this, 4 μg of total DNA and, in parallel, 10 μl of the lipofectamine 2000 reagent were both mixed with 300 μl of FCS- and antibiotics-free medium and incubated for 5 min at Rt. Thereafter, the two solutions were mixed and incubated for 20 min at Rt to allow the formation of DNA-lipofectamine complexes. Then, the DNA-lipofectamine mixture was added dropwise directly onto the cells. After incubation for 4-6 h at 37°C, the transfection solution was replaced by fresh medium. At 12-72 h post-transfection, cells were fixed and permeabilized for use in indirect immunofluorescence staining.

D-6.3 Infection of cultured cells

One day prior to infection, HFFs were seeded in appropriate cell culture vessels suitable in size for the respective experiments: 4 x 10^5 cells per well of a 6-well plate grown on coverslips for indirect immunofluorescence analysis; 3.6 x 10^6 cells per flask for coimmunoprecipitation analysis; 1 x 10^5 cells per 2-well chambered coverglass units with coverslip quality glass bottoms (Lab-Tek, Nunc, Roskilde, Denmark) for confocal time-lapse microscopy. For infection with HCMV AD169 or recombinant HCMVs, the medium of HFFs was replaced by infectious solutions of stock virus (multiplicities of infection between 0.1 and 1; 1 ml per well of a 6-well plate; 5 ml per flask; 0.5 ml per well of chambered coverglass unit) as indicated for the individual experiments. After incubation for 90 min, the supernatant was substituted with fresh medium. At 0-120 h post-infection (hpi), cells were harvested and further processed in the respective experiment.
D-7 Western blot analysis

For the detection of proteins derived from coimmunoprecipitation analysis or *in vitro* kinase assay, protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) by electroblotting under Western blotting buffer at 200 mA for 1 h. Thereafter, the membranes were reversibly stained with Ponceau S solution (Salinovich and Montelaro, 1986) to highlight bands of a standard molecular weight marker (SDS6H2; Sigma-Aldrich, Deisenhofen, Germany). In other cases, when a prestained molecular weight marker was used (Precision Plus Protein Dual Color Standards; Bio-Rad Laboratories GmbH, München, Germany), membranes were directly saturated in 5% skim milk powder solution (J. M. Gabler Saliter GmbH & Co. KG, Obergünzburg, Germany; dissolved in PBSo/0.1% Tween) for at least 2 h at Rt in order to avoid unspecific binding of antibodies. Then, incubation with the primary antibody, dissolved in 3 ml 2.5% skim milk powder solution, was performed for at least 3 h at Rt or overnight at 4°C. After threefold washing of the membranes in PBSo/0.1% Tween for 10 min, incubation with the respective HRP-coupled secondary antibody occurred for 1 h at Rt. After further washing steps and incubation of the membrane in freshly prepared ECL solution (10 ml ECL solution A, 100 µl ECL solution B and 3.1 µl H₂O₂) for 30 sec, detection of the proteins was achieved by the use of the FUJIFILM Luminescent Image Analyzer LAS-1000 (FUJIFILM Europe GmbH, Düsseldorf, Germany). Hereby, the luminol of the ECL solution was converted by the HRP under release of light, which could be detected by the camera (chemiluminescence reaction). If required, the antibodies were removed from the membranes via incubation in Roti®-Free stripping buffer (Roth, Karlsruhe, Germany) at 55°C for 20 min. After extensive washing in PBSo/0.1% Tween, the membranes were stained a second time as described above. AIDA software (version 3.10.039; raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany) was used for optional quantification of relative signal intensities by densitometry.

D-8 Indirect immunofluorescence analysis

HeLa cells or primary HFFs were grown on coverslips for transient plasmid transfection or HCMV infection, respectively. In infection experiments, 0.5 µM to 2 µM of protein kinase inhibitors Gö6976 or AG490 were optionally added at 48 hpi as indicated in the respective experiments. At indicated time points, cells were washed with PBSo following fixation by incubating the cells with a 4% paraformaldehyde solution for at least 10 min at Rt. Then, the cells were washed two times. Permeabilization of the cells was achieved by incubation with 0.2% Triton X-100 in PBSo at 4°C for 20 min. Cells were washed fivefold before the incubation
with 2 mg/ml human γ-globulin (cohn fraction II; Sigma Aldrich, Deisenhofen, Germany) as blocking solution was performed (30 min, 37°C). Thereafter, the cells were incubated with 150 μl of the appropriate primary antibody diluted in PBSO for 90 min at 37°C followed by threefold washing and incubation with 150 μl of the corresponding fluorescent dye-conjugated secondary antibody (diluted in PBSO; 60 min, 37°C). Cells were mounted using the DAPI-containing (4',6-diamidino-2-phenylindole) Vectashield mounting medium (Alexis, Grünberg, Germany) and analyzed with a Zeiss Axiovert-135 inverted fluorescence microscope (Zeiss, Jena, Germany) or the DMI6000 B microscope using a 63x HCX PL APO CS oil immersion objective lens (Leica GmbH, Wetzlar, Germany). Confocal laser-scanning microscopy was performed with a TCS SP5 microscope (Leica GmbH, Wetzlar, Germany). The Meta-Imaging series (MetaVue; Universal Imaging Cop., Downington, PA, USA), LAS AF software (version 1.8.2 build 1465; Leica GmbH, Wetzlar, Germany) and Adobe Photoshop (version 8.0.1; Adobe Systems Incorporated, San Jose, CA, USA) were used for processing of the images.

D-9 Time-lapse microscopy of living cells

HFFs were cultured in 2-well chambered coverglass units with coverslip quality glass bottoms (Lab-Tek, Nunc, Roskilde, Denmark) and infected with recombinant TB40 UL32-EGFP virus at a multiplicity of infection (MOI) of 1.0. At 62 hpi, the cells were washed with Hanks' balanced salt solution with calcium and magnesium (Invitrogen, Karlsruhe, Germany) and then incubated with prewarmed staining solution for live cell endoplasmic reticulum labeling (ER-Tracker red dye; Invitrogen, Karlsruhe, Germany) at a concentration of 1 μM for 20 min at 37°C. After replacing the staining solution with fresh probe-free medium, the living cells were examined using a TCS SP5 confocal laser scanning microscope under the indicated time-lapse conditions. The intracellular trafficking velocity of viral particles was determined by using LAS AF software (version 1.8.2 build 1465; Leica GmbH, Wetzlar, Germany).

D-10 Analysis of protein-protein interactions

D-10.1 Yeast two-hybrid analysis

Yeast cells were transfected by the lithium acetate method (Gietz et al., 1992). Hereby, 5 ml of YAPD medium were inoculated with 50 μl of Saccharomyces cerevisiae strain Y153 and grown overnight at 30°C and 180 rpm. Reeyed salmon testes DNA, which was used as carrier substance, was denatured (95°C, 10 min) and quenched on ice. The Y153 culture was centrifuged and washed with 5 ml of H2O (2200 rpm, 5 min, Rt) followed by resuspending of the cell pellet in 500 μl of a lithium acetate solution (LiAc; 0.2 M) and incubation for 60 min at 30°C.
In the meantime, total DNA of 2 μg was prepared with 10 μl of the denatured carrier substance before 140 μl of a 50% PEG solution was added and the mixture was incubated for further 60 min at 30°C. Then, a heat shock (42°C, 5 min) was performed and subsequent to cooling to Rt, the transfection reaction was mixed with 500 μl H2O. Thereafter, the cells were pelleted by centrifugation (2000 rpm, 2 min) and washed in 1 ml H2O (2000 rpm, 2 min). In a final step, the transfected cells were resuspended in 100 μl H2O and plated on WL-plates. Plates were incubated at 30°C for 3 days to allow selective growth of transfectants. Protein interactions were analyzed using GAL4 fusion proteins. Thereby, protein A fused to the GAL4 activation domain (AD) and protein B fused to the GAL4 binding domain (BD) were coexpressed. In the case of a direct interaction, the AD and BD of the GAL4 transcription factor were brought into spatial proximity resulting in the activation of the GAL4 responsive promoter. Thus transcription of the reporter enzyme β-galactosidase was induced. The activity of β-galactosidase was then determined by filter-lift tests (Breeden and Nasmyth, 1985). Therefore, the transformed colonies were transferred onto a nitrocellulose membrane Hybond™-C-Extra (GE Healthcare, Freiburg, Germany) and subsequently permeabilized by incubation for 1 min in liquid nitrogen. After direct thawing, the membrane was put on a layer of Whatman paper which was soaked in Xgal solution (buffer Z, 1 mM β-mercaptoethanol, 1.5 mM Xgal) and then incubated at 30°C. In the case of protein-protein interactions, β-galactosidase was expressed and able to cleave the Xgal substrate staining the yeast cells blue. Depending on the development of signals (i.e. high or low affinity of interaction), the membranes were incubated for 8-24 h, then dried and analyzed.

**D-10.2 Coimmunoprecipitation analysis**

One day prior to the start of a coimmunoprecipitation (CoIP) assay, 50 mg/ml protein A sepharose was incubated for swelling in CoIP buffer (without protease inhibitors) at 4°C for at least 1 h. The protein A sepharose beads were then supplemented with the respective antibody (2-4 μl antibody in 150 μl of the protein A sepharose/CoIP buffer mix per sample) and incubated overnight at 4°C. The next day, transfected 293T cells or HCMV-infected HFFs were harvested and washed in 10 ml of cold PBSo (2000 rpm, 5 min) before cells were lysed in 500 μl of CoIP buffer supplemented with protease inhibitors, incubated for 20 min on ice and centrifuged (14000 rpm, 10 min, 4°C). Thereafter, 50 μl of each lysate supernatant was boiled with 50 μl 2x SDS sample buffer (95°C, 10 min) for use as expression controls. The remaining volumes of the lysate supernatants (~ 450 μl) were incubated for 30 min at 4°C with rabbit preimmune serum- or unspecific mouse monoclonal antibody-loaded protein A sepharose in order to reduce unspecific binding of proteins to the antibody/protein A sepharose mixtures. After centrifugation (10000 rpm, 2 min, 4°C), these pretreated supernatants were added to protein A sepharose beads loaded with the respective precipitation antibody, which had been
washed three times in CoIP buffer before use (10000 rpm, 2 min, 4°C). After incubation for 1.5 h at 4°C, the CoIP samples were centrifuged (10000 rpm, 2 min, 4°C) and washed five times in CoIP buffer (10000 rpm, 2 min, 4°C). Finally, the immunoprecipitates were recovered by boiling in 30 µl 4x SDS sample buffer (95°C, 10 min) and were subjected to SDS-PAGE and Western blot analysis. In parallel, the expression control samples were analyzed under identical SDS-PAGE/Western blot conditions to visualize a reliable expression level of all proteins.

**D-11 In vitro kinase assay**

For the analysis of the putative phosphorylation of pUL50 by PKCα, both, truncated pUL50 and PKCα were recombinantly expressed in 293T cells. Two days post transfection, immunoprecipitation of PKCα was performed as described in D-10.2 with the exception that the final washing steps of the immunoprecipitates were conducted in PKC IVKA washing buffer. The immunoprecipitates were then subjected to *in vitro* kinase assay (IVKA) reaction, i.e. precipitates were incubated for 40 min at Rt in 40 µl PKC IVKA buffer including 2.5 µCi [γ-33P]ATP (Hartmann-Analytic, Braunschweig, Germany), and optionally 15 mM purified histone 2B (H2B; Roche, Mannheim, Germany) as a positive control for substrate phosphorylation. In control settings, 1 mM STP was added as an inhibitor of PKC activity. Phosphorylation reaction was stopped with 15 µl 4x SDS sample buffer (95°C, 10 min) and samples were analyzed by SDS-PAGE and Western blot procedure. Finally, radioactive phosphorylation signals were detected by exposure of the membranes to phosphorimager plates which were then utilized by the phosphorimager BAS-2000 (Fuji Film. Co., Tokyo, Japan) and evaluated with the AIDA software (version 3.10.039; Raytest Isotopenmessgeraete GmbH, Straubenhartd, Germany).

**D-12 Bioinformatics**

The use of modern bioinformatics tools, in particular the modeling and refinement of the model as well as the visualization (D-12.3) were performed in collaboration with Prof. H. Sticht at the Institute of Biochemistry, Division of Bioinformatics (Erlangen, Germany).

**D-12.1 Protein sequence alignment**

In order to perform amino acid sequence comparisons of the herpesviral nuclear egress proteins, the sequences of a total of 22 members of the UL34 and UL31 families were selected from the NCBI database Entrez Protein (http://www.ncbi.nlm.nih.gov/protein) with the accession numbers as follows:
Material and Methods

<table>
<thead>
<tr>
<th>herpesvirus strain</th>
<th>UL34 family</th>
<th>UL31 family</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 strain 17:</td>
<td>UL34 (P10218)</td>
<td>UL31 (P10215)</td>
</tr>
<tr>
<td>HSV-2 strain HG52:</td>
<td>UL34 (P89457)</td>
<td>UL31 (P89454)</td>
</tr>
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<td>PrV strain Kaplan:</td>
<td>UL34 (Q9ICS7)</td>
<td>UL31 (Q911V7)</td>
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<td>VZV strain HG52:</td>
<td>ORF24 (P09280)</td>
<td>ORF27 (P09283)</td>
</tr>
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<td>HCMV strain AD169:</td>
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<td>UL53 (P16794)</td>
</tr>
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<td>MCMV strain Smith:</td>
<td>M50 (YP_214063)</td>
<td>M53 (YP_214066)</td>
</tr>
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<td>HHV-6A strain Uganda:</td>
<td>U34 (P52465)</td>
<td>U37 (P28865)</td>
</tr>
<tr>
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<td>U34 (Q9QJ35)</td>
<td>U37 (Q9WT27)</td>
</tr>
<tr>
<td>HHV-7 strain JI:</td>
<td>U34 (P52466)</td>
<td>U37 (P52361)</td>
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<tr>
<td>EBV strain B95-8:</td>
<td>BFRF1 (P03185)</td>
<td>BFLF2 (P03183)</td>
</tr>
<tr>
<td>HHV-8 strain GK18:</td>
<td>ORF67 (Q76RF3)</td>
<td>ORF69 (Q77Q39)</td>
</tr>
</tbody>
</table>

Using the ClustalW algorithm (Thompson et al., 1994) by AlignX (component of Vector NTI Advance 9.1.0; Invitrogen, Karlsruhe, Germany), pairwise and multiple alignments of the full amino acid sequences or short regions were performed.

D-12.2 Secondary structure prediction

A secondary structure consensus prediction of pUL50 was performed at the NPS@ server (Network Protein Sequence Analysis; Combet et al., 2000) using several independent methods: DSC (discrimination of secondary structure class; King and Sternberg, 1996), MLRC (multivariate linear regression combination; Guermeur et al., 1999) and PHD (profile network from Heidelberg; Rost and Sander 1993). Transmembrane helices were predicted based on a hidden Markov model at the TMHMM server version 2.0b (Center for Biological Sequence Analysis, Lyngby, Denmark) and potential globular domains by Deleage/Roux definition were assessed by GlobPlot 2.3 (predictor of intrinsic protein disorder & globularity; Linding et al., 2003). Nuclear localization signals (NLS) were identified by the program predictNLS (Cokol et al., 2000).

D-12.3 Search for potential interaction partners of lamin A/C

Candidates for functional protein interaction motifs within lamin A/C were identified in the eukaryotic linear motif (ELM) resource for functional sites in proteins (Puntervoll et al., 2003) using the algorithm from Dinkel and Sticht (Dinkel and Sticht, 2007). The structure of lamin A in complex with Pin1 was modeled based on the known crystal structure of Pin1 in complex with a peptide from the RNA polymerase II C-terminal domain (protein data bank code: 1f8a) (Verdecia et al., 2000). For this purpose, the C-terminal domain ligand sequence was replaced with that of lamin A using the lowest energy rotamers for the non-conserved amino acid side
chains. The complex was subsequently refined by 100 steps of energy minimization using SYBYL 7.3 software (Tripos, St. Louis, MO, USA). Structural analysis and visualization were performed using the program DS ViewerPro (Accelrys Inc., San Diego, CA, USA).
E Results

E-1 Characterization of a nuclear lamina-associated protein complex composed of viral and cellular proteins

E-1.1 Sequence alignment of conserved herpesviral proteins essential for nuclear capsid egress

The translocation of herpesviral capsids from the nucleus to the cytoplasm is dependent on a first budding step at the inner nuclear membrane (INM), which provides the capsids with a primary envelope. For this process, denoted as the nuclear capsid egress, two virally encoded proteins are essential (Mettenleiter T. C., 2004). In the case of HCMV, these are the products of the UL50 and UL53 genes (Dunn et al., 2003). Interestingly, proteins with homology to HCMV pUL50 and pUL53 are present in members of all three herpesvirus subfamilies indicating that their function is conserved throughout the Herpesviridae. Therefore, the homologous proteins are classified into the UL34 and UL31 homology groups (named after the respective HSV-1 genes; Mettenleiter et al., 2006). To further characterize the relationship between amino acid sequence and protein function, a bioinformatical comparison of representatives of both homology groups in human herpesviruses (HSV-1, HSV-2, VZV, HCMV, HHV-6A, HHV-6B, HHV-7, EBV and HHV-8) and animal herpesviruses (MCMV and PrV) was performed. As expected, the highest similarity of pUL50 homologs or pUL53 homologs, respectively, was detected by pairwise alignment of the corresponding sequences within the members of a subfamily. The percentage of completely conserved residues among the sequences of β-herpesvirus pUL50 homologs was 38.7%, and among all herpesvirus homologs 22.9% (data not shown). pUL53 homologs revealed a slightly higher conservation, with 44.3% among β-Herpesvirinae compared to 25.5% among Herpesviridae (data not shown). However, the homology of pUL50 as well as of pUL53 was not equally distributed along the sequence. Therefore, multiple sequence alignments of members of β-Herpesvirinae or Herpesviridae were generated. For pUL50, a variable C-terminal region (non-conserved region, NCR\textsubscript{UL50}) and two conserved regions (CR1\textsubscript{UL50} and CR2\textsubscript{UL50}) within the N-terminus were identified (Table 1). Consensus sequences within CR1\textsubscript{UL50} and CR2\textsubscript{UL50} revealed conservation in more than 90% of the corresponding regions among β-Herpesvirinae and more than 40% among Herpesviridae. Within CR1/2\textsubscript{UL50}, more than 30% (β-Herpesvirinae) or 2% (Herpesviridae) of the residues were identical and were found in all analyzed sequences. The homology towards the consensus sequence of NCR\textsubscript{UL50} was in both cases below 20% with no highly conserved residues.
TABLE 1. Results of a multiple protein sequence alignment of pUL50 homologs within \( \beta \)-Herpesvirinae and Herpesviridae. Consensus and identity positions are denoted in \% (see D-12.1 for used algorithm). \( \beta \)-Herpesvirinae, HCMV, MCMV, HHV-6A/B and HHV-7; Herpesviridae, HSV-1, HSV-2, PrV, VZV, HCMV, MCMV, HHV-6A/B, HHV-7, EBV and HHV-8. CR/NCR, conserved/non-conserved region; aa, amino acids.

<table>
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<th>region of HCMV pUL50</th>
<th>CR(^{\text{UL50}}) (aa 1-94)</th>
<th>CR(^{\text{UL50}}) (aa 100-181)</th>
<th>NCR(^{\text{UL50}}) (aa 182-397)</th>
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<td>Herpesviridae</td>
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</table>

\(^{1}\) percentage of amino acid positions identical with the consensus sequence of the sequences analyzed
\(^{2}\) percentage of amino acid positions identical among all sequences analyzed

Similarly to the comparison of the pUL50 sequences, multiple alignments of pUL53 sequences discovered a variable N-terminal region (NCR\(^{\text{UL53}}\)) and four conserved regions (CR\(^{\text{UL53}}\) to CR\(^{\text{UL53}}\)) (Table 2). These regions correspond to the conserved and variable regions previously identified among 36 homologs of pUL53 by Lötzerich et al. (2006). Consensus sequences of CR\(^{\text{UL53}}\) to CR\(^{\text{UL53}}\) revealed conservation in more than 88\% of the corresponding regions among \( \beta \)-Herpesvirinae and more than 39\% among Herpesviridae. More than 44\% of the residues within CR1/2\(^{\text{UL53}}\) or 25\% within CR3/4\(^{\text{UL53}}\), respectively, were identical among \( \beta \)-Herpesvirinae. In Herpesviridae, only CR1\(^{\text{UL53}}\) and CR3\(^{\text{UL53}}\) possessed a number of fully identical residues (7.4\% and 2.1\%, respectively). The homology towards the consensus sequence of NCR\(^{\text{UL53}}\) was less than 39\% (\( \beta \)-Herpesvirinae) or less than 13\% (Herpesviridae),

TABLE 2. Results of a multiple protein sequence alignment of pUL53 homologs within \( \beta \)-Herpesvirinae and Herpesviridae. Consensus and identity positions are denoted in \% (see D-12.1 for used algorithm). \( \beta \)-Herpesvirinae, HCMV, MCMV, HHV-6A/B and HHV-7; Herpesviridae, HSV-1, HSV-2, PrV, VZV, HCMV, MCMV, HHV-6A/B, HHV-7, EBV and HHV-8. CR/NCR, conserved/non-conserved region; aa, amino acids.

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<th>CR(^{\text{UL53}}) (aa 163-243)</th>
<th>CR(^{\text{UL53}}) (aa 253-282)</th>
<th>NCR(^{\text{UL53}}) (aa 1-57)</th>
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\(^{1}\) percentage of amino acid positions identical with the consensus sequence of the sequences analyzed
\(^{2}\) percentage of amino acid positions identical among all sequences analyzed
with only highly conserved residues among \( \beta \)-Herpesvirinae (3.2%). The long C-terminal tail (amino acids 283-376) is unique for HCMV pUL53 and was therefore not aligned individually with other sequences. In summary, HCMV pUL50 and pUL53 both possess conserved regions with high homology or even identity compared to their herpesviral homologs, indicating at least a partial functional conservation.

**E-1.2 Analysis of the interaction between pUL50 and pUL53 in transiently transfected cells**

**E-1.2.1 Recruitment of pUL53 to the nuclear envelope by pUL50**

In the following, HCMV pUL50 and pUL53 were further characterized in respect of their intracellular localization. First, *in silico* analysis of the primary sequences of pUL50 and pUL53 predicted a putative transmembrane domain of pUL50 within amino acids 359-381, whereas no region with probability of membrane localization was detected for pUL53 (Fig. 3A). Furthermore, a putative nuclear localization signal (NLS) was predicted only for pUL53 (amino acids 13-24), but no NLS was found within pUL50 (data not shown). Second, the intracellular localization of both proteins was investigated by immunofluorescence analysis (Fig. 3B). Consistent with the predicted transmembrane domain, transiently overexpressed pUL50 was tightly associated with the nuclear envelope (nuclear rim staining; Fig. 3B, panels a-d). In contrast to pUL50, pUL53 was evenly distributed throughout the nucleus (Fig. 3B, panels e-h) as expected since an NLS, but no transmembrane domain was predicted for pUL53. Intriguingly, coexpression of pUL50 and pUL53 resulted in a complete relocalization of pUL53 towards a distinct nuclear rim staining perfectly colocalizing with pUL50 (Fig. 3B, panels i-m). Notably, C-terminally truncated pUL50 lacking amino acids 359-397 produced a diffuse nuclear localization and no nuclear envelope association (data not shown), which confirmed the predicted transmembrane domain. Consequently, this deletion mutant failed to influence the localization of pUL53, indicating that the capacity of pUL50 to induce a relocalization of pUL53 is dependent on its association with the nuclear envelope (data not shown; Milbradt *et al.*, 2007). In order to investigate if the recruitment of pUL53 by pUL50 was mediated by direct protein-protein interaction, a coimmunoprecipitation (CoIP) analysis was performed. Hereby, a positive signal was obtained for the interaction of pUL50 and pUL53, whilst interaction of pUL50 with another HCMV protein, pUL97, was negative in a control reaction. The obtained results were confirmed by further CoIP analysis with truncated pUL50 lacking amino acids 359-397 which also interacted strongly with pUL53 (data not shown; Milbradt *et al.*, 2007).
The intracellular localization of coexpressed pUL50 and pUL53 was further analyzed by confocal laser-scanning microscopy (CLSM; Fig. 4). First experiments with this high-resolution microscopy technique revealed that transiently overexpressed pUL50 and pUL53 rather colocalized in speckled aggregations at the nuclear envelope (Fig. 4A, panels a-e) than in a perfect rim staining as observed by wide-field microscopy (Fig. 3B, panels i-m). Moreover, the definite colocalization of pUL50/pUL53 was investigated by costaining with markers of the nuclear lamina as well as those of the nuclear pore complex (NPC). Interestingly, the pUL50/pUL53 speckled fine-structures were located within the nuclear lamina suggested by the partial colocalization with lamin A/C (Fig. 4A, panels f-k) and the lamin B receptor (LBR; Fig. 4A, panels l-p). Hereby, lamin A/C was marginally affected by the expression of pUL50 and pUL53 illustrated by the infrequent occurrence of invaginations of the nuclear lamina (Fig. 4A, panels h and k). These invaginations of the nuclear lamina were not observable in vector transfected cells (Fig. 4B, panel a). Contrary to lamin A/C, localization of the LBR was not affected by pUL50 and pUL53 (compare Fig. 4A, panel m with 4B, panel b). Concerning the speckled pattern of pUL50/pUL53 colocalization, a distribution close to the NPC appeared suggestive (Fig. 4B, panel c). However, pUL50/pUL53 did not colocalize with the NPC (Fig. 4A, panels q-u; note the lack of yellow signals in merge), indicating that the nuclear pores are not the target sites of pUL50 and pUL53.
Results

FIGURE 4. Detailed analysis of the pUL50/pUL53 colocalization. HeLa cells were either cotransfected with expression plasmids coding for tagged versions of pUL50 and pUL53 (A) or transfected with pcDNA3.1 (vector) as control (B). At 2 days post-transfection, cells were subjected to indirect immunofluorescence analysis using indicated antibodies and were subsequently analyzed by confocal laser-scanning microscopy (CLSM). Inset images illustrate enlargements of representative areas of the nuclear envelope.

E-1.3 Yeast two-hybrid analysis of interactions between pUL50, pUL53 and other proteins

Identification of proteins interacting with the viral nuclear egress proteins pUL50 and pUL53 is a prerequisite to elucidate the processes responsible for HCMV nuclear capsid egress. Therefore, the goal of this study was to discover putative interaction partners of pUL50 and pUL53. Thus, plasmids coding for pUL53 and pUL50 fused to either GAL4 AD or GAL4 BD were cotransfected with a selection of putative interaction partners. The results detected in the subsequent yeast two-hybrid analysis are summarized in Table 3. Particularly noteworthy, a deletion mutant encoding amino acids 1-358 of pUL50 was used throughout the whole experiment, because the transmembrane domain appeared to render the full-length pUL50 non-functional in the yeast two-hybrid system. In control settings, none of the proteins produced any background activity guaranteeing the specificity of the detected positive signals (Milbradt et al., 2007, 2009b). In a first step, the direct interaction between pUL50 and pUL53, previously observed by CoIP analysis (Milbradt et al., 2007), was confirmed in the yeast two-hybrid system. Next, the viral protein kinase pUL97 which is required at the stage of nuclear egress (Krosky et al., 2003), was
Results

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considered to interact with pUL50 or pUL53. However, pUL97 did not interact with either of the two nuclear egress proteins. It appeared conceivable, that pUL97 may not directly but indirectly interact with pUL50 or pUL53 via a viral or cellular adaptor protein. A plausible candidate for mediating this interaction was the cellular protein p32 which was demonstrated to recruit pUL97 to the LBR in HCMV-infected cells (Marschall et al., 2005). Actually, pUL50 interacted with p32 in the yeast two-hybrid system, while pUL53 was negative for this interaction (Table 3). In turn, an N-terminal fragment of the LBR (amino acids 1-208) did neither interact with pUL50 nor pUL53. In a next step, cellular protein kinase C (PKC) was taken into account as a further interaction partner of pUL50 or pUL53 since involvement of PKC in the nuclear capsid egress had previously been demonstrated for HSV-1 (Park and Baines, 2006) and MCMV (Muranyi et al., 2002). Interestingly, PKC isoforms ε and ζ were here identified to interact with pUL50 but not with pUL53 (no functional construct was available for testing in parallel PKC isoform α). In a final step, investigating interactions among all tested proteins, p32 was outstanding in interacting with a number of proteins, i.e. confirming the previously reported interactions with pUL97 (Marschall et al., 2005), PKC isoforms (Storz et al., 2000; Robles-Flores et al., 2002) and the LBR (Mylonis et al., 2004). Addressing the issue of specificity, p32 was further investigated and did not interact with a series of cytomegaloviral proteins other than pUL50 or pUL97 (Milbradt et al., 2009b). Particularly noteworthy, several positive interactions observed in this study were detected only in one direction, but not in the reverse setting concerning tagged GAL4 AD and GAL4 BD (Table 3). Importantly, however, the findings were reproducible in several independent experiments. Furthermore, negative results in the yeast two-hybrid system may generally be due to simple limitations such as low expression levels or non-physiological protein folding. Specificity-controlled positive results, in any case, even at low signal intensity, generally argue for direct protein-protein interaction.

TABLE 3. Results of a comparative yeast two-hybrid analysis of interactions between pUL50, pUL53 and other nuclear lamina-associated proteins. Classification of positive or negative protein interactions was obtained by staining of selected yeast clones in a standard filter lift assay. AD/BD, protein fused to GAL4 DNA activation/binding domain; +, blue signal, indicating an interaction; −, white/yellow signal, indicating no interaction; ±, weak blue signal, no clear result; n.d., not determined.

<table>
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<tr>
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<th>AD</th>
<th>BD</th>
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<th>pUL53</th>
<th>pUL97</th>
<th>p32</th>
<th>PKCε</th>
<th>PKCζ</th>
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<tbody>
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</tbody>
</table>
E-1.4 Confirmation of detected protein interactions by coimmunoprecipitation analysis

E-1.4.1 Interaction of pUL50 with recombinantly expressed and endogenous PKCα

PKCα was used for transient overexpression in the mammalian system. For CoIP analysis, PKCα-GFP was coexpressed together with pUL50-HA, pUL53-HA or pUL97-HA followed by immunoprecipitation of the viral proteins by an HA-specific antibody. Notably, immunoprecipitation of pUL50-HA resulted in a distinct coprecipitation of recombinantly expressed PKCα-GFP (Fig. 5A, lane 3), whereas the CoIP was negative in the case when pUL53 or pUL97 was immunoprecipitated (Fig. 5A, lane 4-5). When Western blot detection of the CoIP samples was performed with a monoclonal antibody recognizing both recombinant and endogenous PKCα, two PKCα-specific bands were obtained, with the recombinant form being dominant over a faint band for endogenous PKCα (Fig. 5A, lane 8). The lack of any background in samples with no expression of HA-tagged proteins (Fig. 5A, lanes 1-2, 6-7) or samples immunoprecipitated with an unspecific preimmune serum (Fig. 5A, lanes 11-15) demonstrated a high specificity of the CoIP. Protein expression levels were monitored by lysate

FIGURE 5. Interaction of pUL50 with recombinant and endogenous PKCα. 293T cells were either cotransfected with expression plasmids coding for tagged versions of pUL50, pUL53 or pUL97 together with PKCα or single-transfected with an expression plasmid encoding the red fluorescent protein (RFP) as control. At 2 days post-transfection, cells were lysed and immunoprecipitated using pAb-HA or an unspecific preimmune serum as indicated. Thereafter, immunoprecipitates were subjected to Western blot analysis using indicated antibodies (A). Expression of proteins was controlled via tag- or protein-specific antibodies (B). Ig-HC, cross-reactive band for immunoglobulin heavy chain.
controls taken prior to the addition of the CoIP antibody (Fig. 5B). Thus, the combined data obtained by CoIP and yeast two-hybrid analyses suggested that pUL50 interacts with several isoforms of PKC (including α, ε and ζ).

**E-1.4.2 Direct phosphorylation of pUL50 by PKCα**

The finding of a protein-protein interaction between pUL50 and PKC raised the question whether pUL50 is a substrate of PKC-mediated phosphorylation. Potential phosphorylation of pUL50 by PKC was addressed by *in vitro* kinase assays (IVKA) using PKCα and putative substrate proteins from transiently transfected cells. In a first step, pUL50-HA, pUL53-HA and PKCα-GFP were expressed separately, immunoprecipitated and combined in IVKA reactions (2fold excess of substrate precipitates compared with kinase precipitates). Several weakly phosphorylated protein species were detected (data not shown). Since the corresponding protein bands potentially comigrated with pUL50-HA and pUL53-HA, this IVKA did not allow a conclusive interpretation. Therefore, an improved setting was performed using a combined CoIP/IVKA strategy in a second step (Fig. 6). PKCα and putative substrates, such as pUL50(1-358)-HA, were coexpressed with PKCα-GFP and coimmunoprecipitated using an HA-specific antibody. In this case, phosphorylation of pUL50(1-358)-HA by PKCα-GFP was detected (Fig. 6A, lane 5). A prerequisite for this positive phosphorylation signal was the successful CoIP of PKCα-GFP by pUL50(1-358)-HA (Fig. 6B, lower panel, lane 5). However, pUL53-HA did not coimmunoprecipitate PKCα-GFP as expected (Fig. 6B, lower panel, lane 6) and therefore no phosphorylation of pUL53-HA was detected with this assay (Fig. 6A, lane 6). Interestingly, CoIP of a protein complex including pUL50-HA, pUL53-HA and PKCα-GFP resulted in a weaker phosphorylation signal (Fig. 6A, lane 7). A reasonable explanation is given by less amounts of coimmunoprecipitated PKCα-GFP (Fig. 6B, lower panel, lane 7 compared to lane 5), since pUL53 and PKCα were likely to compete for the binding to pUL50. Moreover, autophosphorylation of PKCα-GFP was below the detection limit (Fig. 6A, lanes 5 and 7). Notably, the strongest signals for *in vitro* phosphorylation of the standard substrate histone 2B (H2B) were demonstrated for those samples containing PKCα-GFP (Fig. 6A, lanes 5 and 7). Other detectable signals for H2B phosphorylation (Fig. 6A, lanes 1-3) and also for individually expressed pUL50(1-358)-HA (Fig. 6A, lane 2) most probably resulted from the coimmunoprecipitation of endogenous PKC or an unknown associated protein kinase. As a specificity control, a known inhibitor of PKC activity, staurosporine (STP) was applied to the reactions resulting in complete suppression of phosphorylation signals for pUL50(1-358)-HA and H2B (Fig. 6A, lanes 8 and 9). Thus, phosphorylation of pUL50 was detectable *in vitro* and PKC is the most probable candidate for this activity.
FIGURE 6. Direct phosphorylation of pUL50 by PKCa in vitro. (A) pUL50(1-358)-HA, pUL53-HA and PKCa-GFP were coexpressed in 293T cells as indicated. At 2 days post-transfection, cells were lysed, immunoprecipitated using pAb-HA and subjected to an IVKA. H2B was added to all reactions as a standard kinase substrate. In control samples, PKC activity was inhibited by STP (lanes 8-9). Phosphorylation signals of the IVKA blot were detected by phosphorimager exposure. (B) Successful immunoprecipitation was controlled by Western blot immunostaining of the IVKA blot with mAb-HA and mAb-GFP as indicated.

E-1.4.3 Interaction of pUL50, pUL53 and pUL97 with cellular p32

Since the interaction between pUL50 and the cellular pUL97 binding protein p32 was detected in the yeast two-hybrid system (Table 3), confirmation was addressed in the mammalian system. For this, the interaction capacity of p32 with several herpesvirus-encoded proteins was investigated by CoIP analysis (Fig. 7A). Positive CoIP signals for p32 interaction were obtained with full-length pUL50 and a C-terminally truncated pUL50 mutant (lacking the transmembrane domain), pUL97 and pUL53 (Fig 7A, upper panel, lanes 3-6). No positive CoIP signals were obtained for N-terminally truncated pUL97 (lacking the p32 interaction region; Marschall et al., 2005) and for the HSV-1 protein kinase UL13, applied as a control (Fig. 7A, upper panel, lanes 7 and 8). Concerning the interaction of p32 with pUL50 variants and pUL97, no
background was detected with an unspecific preimmune serum (Fig 7A, upper panel, lanes 9-10, 12-14). However, in the case of pUL53, a background signal was observable though it was weaker than the specific CoIP signal (Fig 7A, upper panel, lane 11 compared to lane 5). For this reason, and because the interaction between p32 and pUL53 was unexpected since it was not detected by the yeast two-hybrid analysis (Table 3), confirmation by a reverse CoIP assay was performed (Fig. 7B). FLAG-tagged p32 was hereby immunoprecipitated and a CoIP signal of pUL53 was detected (Fig. 7B, upper panel, lane 3). This time, the lack of any background in samples immunoprecipitated with an unspecific preimmune serum (Fig. 7B, upper panel, lane 5) supported the specificity of the interaction. In both CoIP analyses, the successful immunoprecipitation and expression of proteins was ensured by immunostaining the CoIP (precipitation control) and expression control blots with tag-specific antibodies (Fig. 7A and B, middle and lower panels).

FIGURE 7. Interaction of p32 with pUL50, pUL53 and pUL97. (A) 293T cells were cotransfected with expression plasmids coding for p32-FLAG and HA-tagged versions of putative viral interaction partners. At 2 days post-transfection, the cells were lysed and immunoprecipitated using pAb-HA (lanes 1-8) or unspecific preimmune serum (lanes 9-14). Thereafter, immunoprecipitates were subjected to Western blot analysis using indicated antibodies. Successful immunoprecipitation and expression levels were presented via tag-specific antibodies as indicated. (B) Confirmation of the interaction between pUL53 and p32. CoIP analysis was performed with an inverted application of tag-specific antibodies as in (A). pre, preimmune serum.

In regard to the interaction of pUL53 with p32, the discrepancy between the results from these CoIP analyses and the yeast two-hybrid study may arise from irregular folding of one or both of the proteins in yeast cells. On the other hand, the yeast two-hybrid technique is able to discriminate between direct and indirect interaction between two proteins in most cases.
Therefore, the difference may also be because a further cellular protein is required for the interaction of pUL53 with p32, which may be present in mammalian but not in yeast cells.

**E-1.4.4 Association of pUL50 and pUL53 with the nuclear lamina by interaction of p32 with the LBR**

Yeast two-hybrid and CoIP data obtained so far suggested the organization of an interaction network between pUL50, pUL53, pUL97, p32 and PKC. Additionally, p32 is associated with the nuclear lamina by interacting with the LBR (Marschall et al., 2005). Thus, CoIP analysis was performed to identify further interaction partners of the LBR (Fig. 8). The above mentioned proteins were therefore expressed individually and used for a LBR-specific CoIP experiment. Notably, Western blot staining detected the coimmunoprecipitated endogenous LBR. A positive CoIP signal of LBR was detected with p32 (Fig. 8A, lane 7), but was negative for pUL50, pUL53, pUL97 and PKCα (Fig. 8A, lane 2-6). The HCMV tegument protein pUL26 served as a negative control (Fig. 8A, lane 8), since for pUL26 neither involvement in the nuclear capsid

![Image](image-url)
egress nor nuclear lamina association had been described (Lorz et al., 2006). Specificity controls with mAb-MYC (Fig. 8A, lanes 9-14) and expression controls (Fig. 8B) confirmed the reliability of this experiment. Hence, due to several protein-protein interactions with the viral nuclear egress proteins as well as with viral and cellular protein kinases, p32 appeared to be a central adaptor protein mediating the contact of its interaction partners to the nuclear lamina by binding the LBR.

### E-1.5 Recruitment of the viral protein kinase pUL97 and cellular PKCα to the nuclear envelope by pUL50

On the basis of the CoIP data, immunofluorescence analysis was performed in order to assess the effect of protein-protein interactions on the intracellular localization. The relocalization of pUL53 by its interaction partner pUL50 was previously illustrated in Fig. 3. Another interaction partner of pUL50 was identified in PKCα (Fig. 5), which was distributed mainly throughout the cytoplasm when individually expressed in HeLa cells (Fig. 9B, panel a). Importantly, coexpression with pUL50 relocalized PKCα towards the nuclear rim (Fig. 9A, panels a-d). In contrast to full-length pUL50, truncated pUL50(1-358), which lacks the transmembrane domain, did not produce a relocalization of PKCα (Fig. 9A, panels e-h). Since pUL50(1-358) still coimmunoprecipitated PKCα (Fig. 6B, lower panel), it appeared that the nuclear rim localization of pUL50 was necessary for the relocalization of PKCα. Further analysis revealed, that pUL50 coexpression resulted surprisingly in a partial recruitment of pUL97 to distinct pUL50 speckles at the nuclear rim (compare Fig. 9A, panels i-m with 9B, panel c), though no direct interaction between both proteins was detected before. To explain the pUL50-mediated recruitment of pUL97, the localization of p32 was analyzed since p32 interacts with pUL50 and pUL97. Specifically, no relocalization of FLAG-tagged full-length p32 was observed when coexpressed with pUL50 (data not shown). Particularly noteworthy, endogenous p32 is translated as a precursor protein from which an N-terminal mitochondrial localization signal (MLS) is cleaved off to create the mature p32. Furthermore, tagging the full-length p32 might interfere with the proteolytic removal of the MLS (Brokstad et al., 2001; Wang et al., 1997). To mimic the endogenous protein, an N-terminally truncated p32 lacking amino acids 1-49 was used for further experiments since this construct possesses no functional MLS. As expected, this construct exhibited no localization typical for mitochondria but was diffusely located in the cytoplasm and slightly in the nucleus (Fig. 9B, panel d). Interestingly, this N-terminally truncated p32 accumulated at the nuclear lamina when coexpressed with pUL50 and pUL97 (Fig. 9A, panels n-q). Therefore, it appeared reasonable that the previous identified recruitment of pUL97 by p32 to the LBR (Marschall et al., 2005) might be increased by pUL50. On the other hand,
pUL50 could direct the pUL97-p32 complex to different target sites at the nuclear lamina by its own localization anchored in the nuclear membrane.

FIGURE 9. Recruitment of pUL97 and PKCa to the nuclear envelope mediated by pUL50. HeLa cells were cotransfected (A) or transfected individually (B) with constructs expressing pUL50-HA, PKCa-GFP, pUL97-F, and/or p32(50-282)-F. At 2 days post-transfection, cells were fixed and immunostained with tag-specific antibodies as indicated. Samples were analyzed by either standard wide-field microscopy (A, a-h and n-q; B, a-b and d) or CLSM (A, i-m; B, c). The merge of signals is shown on the right of each staining. Cell nuclei were counterstained with DAPI.

E-1.6 Detection of pUL50-associated protein complexes in transiently transfected and HCMV-infected cells

The data obtained from CoIP (E-1.4) and immunofluorescence analyses (E-1.5) provided initial evidence for the establishment of an interaction network between cytomegaloviral and cellular proteins. Those proteins of this interaction network that do not directly interact (e.g. pUL53 and PKCa) are very likely to be linked in a multi-protein complex through indirect interactions. In order to address this point, triple-transfection with expression constructs coding for pUL50, pUL53 and PKCa each carrying an individual tag for CoIP analysis was applied (Fig. 10A). Strikingly, PKCa was indirectly coimmunoprecipitated by a pUL53-directed antibody (mAb-FLAG; Fig. 10A, upper panel, lane 8), but not by an unrelated antibody (mAb-MYC; Fig. 10A, upper panel, lane 9). All immunoprecipitates obtained from single- or double-transfected controls (Fig. 10A, lanes 1-7), particularly pUL53 and PKCa without pUL50
(Fig. 10A, lane 6), were negative for PKCα. In a similar approach, the indirect interaction between pUL53 and PKCα was investigated in HCMV-infected primary fibroblasts. Lysates were prepared at a late time point of infection (3 days) and were subjected to CoIP with mAb-PKCα (Fig. 10B). Similar to the CoIP of transfected cells, pUL53 was specifically detected in the immunoprecipitates in cells infected with a multiplicity of infection (MOI) of 1.0 (Fig. 10B, upper panel, lane 3). In cells infected under low-MOI conditions (MOI of 0.1), the amount of coimmunoprecipitated pUL53 was below the detection limit of this assay (Fig. 10B, upper panel, lane 2). In addition, specificity of the CoIP was controlled in uninfected cells (Fig. 10B, lane 1 and 4), and by the use of an unrelated CoIP-antibody (pAb-calreticulin) resulting in the lack of any background bands (Fig. 10B, upper panel, lanes 4-6). Here again, the CoIP of pUL53 by a PKCα-specific antibody in HCMV-infected cells, though pUL53 did not directly interact in various assay systems (yeast two-hybrid and CoIP analyses), appeared to be bridged by pUL50 (Fig. 10C). This finding is consistent with the model of a multiply linked protein complex which includes at least pUL50, pUL53 and PKCα and possibly further components.

![FIGURE 10. Analysis of putative pUL50-associated protein complexes from transfected and HCMV-infected cells.](image-url)

(A) 293T cells were transfected with constructs encoding tagged versions of pUL50, pUL53 and/or PKCα (HA, FLAG or GFP, respectively). CoIP assays were performed at 2 days post-transfection with mAb-FLAG (lanes 1-8) or mAb-MYC (lane 9) and subjected to Western blot analysis. Expression controls were stained with tag-specific antibodies as indicated. (B) HFFs were infected with HCMV strain AD169 at an MOI of 0.1 to 1.0, or remained uninfected (mock). At 3 days post-infection, cells were lysed and used for CoIP analysis with mAb-PKCα (lanes 1-3) or pAb-calreticulin (lanes 4-6). Detection of coimmunoprecipitates (upper panel) and of expression controls (lower panel) was performed by Western blots using pAb-UL53. (C) Illustration of the indirect interaction between pUL53 and PKCα mediated by pUL50.
summary of the identified protein-protein interactions (E-1.3, E-1.4, E-1.6) is presented illustrating the formation of a hypothetical multi-protein complex at the nuclear lamina (Fig. 11). Functional consequences of these interactions and the postulated mechanistic details required for HCMV nuclear egress are discussed in F-3.

**FIGURE 11. Summarizing scheme of identified interactions which favor the formation of a multi-protein complex constituted by HCMV and cellular proteins.** This model represents schematically all relevant non-covalent protein interactions identified so far, indicated by interaction symbols (■). Important substrate protein phosphorylations resulting from the activity of depicted protein kinases, identified in this thesis or in other studies (Marschall et al., 2005; Hamirally et al., 2009), are indicated (P). Further as yet unidentified proteins/protein kinases may additionally be involved (X). black ■, interaction identified by yeast two-hybrid and CoIP analyses; grey ■, interaction identified by only yeast two-hybrid or CoIP analyses, respectively.

**E-1.7 Definition of interaction domains within pUL50 for its three interaction partners**

Previous experiments demonstrated that pUL50 has three interaction partners, namely pUL53, p32 and PKC. Further CoIP analysis additionally illustrated the capability of pUL50 to bridge the interaction between the two non-interacting proteins pUL53 and PKCα (Fig. 10), apparently allowing the simultaneous binding of at least two proteins to pUL50. On this basis, the question about the regions responsible for the interaction of pUL50 with its three interaction partners was addressed.

**E-1.7.1 Construction of truncation mutants based on structural properties of pUL50**

Truncation mutants of pUL50 were generated on the basis of structural properties of pUL50 in order to use them for mapping interaction domains within pUL50 by CoIP analyses. Therefore,
computational analyses were performed with the pUL50 amino acid sequence assigning secondary structure elements to certain regions (Fig. 12). The N-terminus was predicted to be rich in regular secondary structure and to adopt a globular fold, comprising at least residues 45-181. Interestingly, this part of pUL50 corresponded closely to the regions found to be highly conserved among the pUL50 proteins of the \( \beta \)-Herpesvirinae (CR\textsuperscript{UL50}\textsubscript{1} and CR\textsubscript{2}\textsuperscript{UL50}; Table 1). Residues 1-44 and 182-358 were not expected to adopt a defined 3-D structure and were either entirely disordered or formed only short local elements of secondary structure. These findings suggested that the highly conserved globular core of pUL50 represents the major interaction site for the interaction partners, while the less-conserved flanking regions may play a role in

![Structural properties of pUL50. Regions are annotated according to their tendency to adopt secondary structure elements as indicated. Details of the secondary structure prediction are presented in Milbradt et al., 2009b. Arrows designate the boundaries of the cloned deletion constructs.](image-url)
increasing binding affinity and specificity for distinct interaction partners. To dissect the putative roles of the globular domain and the flanking non-globular regions, several truncation mutants were designed (Fig. 12).

**E-1.7.2 Mapping of three interaction domains within pUL50 by CoIP analysis**

Truncation mutants were used to map the domains of pUL50 which are responsible for the interaction with pUL53, PKCα and p32. 293T cells were cotransfected with expression constructs coding for HA-tagged N- and C-terminal truncation mutants of pUL50 together with FLAG-tagged pUL53. In the subsequent CoIP, the pUL50 truncation mutants were immunoprecipitated followed by Western blot detection of potentially coprecipitated pUL53 (Fig. 13A). None of the N-terminally deleted constructs was capable of coimmunoprecipitating pUL53 (Fig. 13A, upper panels, lanes 4-8), whereas C-terminal deletions up to amino acid 250 supported this interaction (Fig. 13A, upper panels, lanes 18-22). In parallel, specificity of the CoIP was verified by results with rabbit preimmune serum (Fig. 13A, upper panels, lanes 9-14 and 23-28). Expression levels of all recombinant proteins were monitored by expression control samples taken prior to the addition of the CoIP antibody (Fig. 13A, lower panels). Since these experiments only allowed a determination of the region required for interaction with pUL53 to be amino acids 1-250 of pUL50, further CoIP analyses were performed. In the case of the N-terminus of pUL50, deletions up to amino acid 10 were tolerable for the interaction (Fig. 13B, upper panel, lanes 4-5), though the CoIP signal compared to full-length pUL50 (Fig. 13B, upper panel, lane 3) was weaker. Noteworthy, the expression controls revealed a lower level of pUL50(15-397)-HA and pUL50(20-397)-HA expression (Fig. 13B, lowest panel, lane 6-7) than that of pUL50(5-397)-HA and pUL50(10-397)-HA (Fig. 13B, lowest panel, lane 4-5). However, the reliability of this experiment was provided by immunostaining the CoIP blot with a pUL50-specific antibody which demonstrated equal amounts of pUL50 mutants in the precipitates (precipitation controls; Fig. 13B, middle panel, lanes 3-7). In order to further narrow down the interaction domain for pUL53 on the C-terminal side, a modified experimental set-up including GFP-tagged pUL50 truncations and a pUL53-specific CoIP antibody was applied (Fig. 13C). Notably, pUL53-F and PKCα-GFP served as negative control to exclude false positive signals due to unspecific binding of the GFP-tag (Fig. 13C, upper panel, lane 2). Importantly, only the C-terminally deleted construct comprising amino acids 1-181 of pUL50 was coimmunoprecipitated by pUL53 (Fig. 13C, upper panel, lane 4), whereas further C-terminal deletions of pUL50 abrogated the interaction with pUL53 (Fig. 13C, lanes 5-8). The latter result was confirmed with an inverted application of tag-specific antibodies as in Fig. 13C (data not shown). Thus, the CoIP data suggested that the region comprising amino acids 1-181 of pUL50 participates in the interaction with pUL53. In particular, amino acids 10-14 were absolutely essential for binding.
FIGURE 13. **Mapping of the pUL53 interaction domain within pUL50 by CoIP analyses.** HA- or GFP-tagged N- and C-terminal truncated versions of pUL50 were transiently coexpressed in 293T cells with FLAG-tagged pUL53 as indicated. At 2 days post-transfection, cells were lysed and the HA-tagged deletion mutants of pUL50 were precipitated using pAb-HA (A) or mAb-HA (B), or the FLAG-tagged pUL53 was precipitated using mAb-FLAG (C). In control settings, preimmune serum was used for CoIP (A, lanes 9-14 and 23-28). Coimmunoprecipitates and expression control samples were subjected to Western blot analysis using tag-specific antibodies as indicated.
In regard to the interaction domains for the other two interaction partners of pUL50, analog CoIP analyses were performed for PKCα and p32 (data not shown). In particular, amino acids 100-280 and 100-358 of pUL50 were required for binding to PKCα and p32, respectively. Details of the determination of interaction domains of pUL50 for binding to PKCα and p32 are presented in Milbradt et al. (2009b). A summary of the results of the conducted experiments is illustrated schematically including the complete CoIP data and the resulting partly overlapping interaction domains within pUL50 for binding to pUL53, PKCα and p32 (Fig. 14). Particularly noteworthy, the region responsible for interaction with pUL53 resembles the highly conserved part of pUL50.

![Image of CoIP data summary]

**FIGURE 14. Schematic summary of three interaction domains within pUL50.** Presentation of CoIP data obtained with N- and C-terminal deletion mutants of pUL50, as well as the determined regions required for interacting with pUL53, PKCα and p32. ‡, strong positive CoIP signal; +, positive CoIP signal; −, negative CoIP signal; ±, weak CoIP signal, no clear result; n.d., not determined.

### E-1.7.3 Failure of N-terminal truncation mutants of pUL50 to recruit pUL53 and PKCα

After the identification of pUL50 regions which were responsible for binding to its interaction partners, truncated versions of pUL50 were analyzed concerning their ability to recruit pUL53 and PKCα in transfected cells (Fig. 15). pUL50 versions expressed as amino acids 20-397 or 150-397 were located similarly at the nuclear rim as full-length pUL50 (Fig. 15B), with pUL50(20-397) being additionally present in the cytoplasm (Fig. 15B, panel b). None of these
RESULTS

FIGURE 15. Interaction with pUL50 necessary for the recruitment of pUL53 and pUL97. HeLa cells were cotransfected (A and C) or transfected individually (B) with pcDNA3.1 (vector) or constructs expressing tagged versions of pUL50, pUL53 or PKCα. At 2 days post-transfection, the cells were subjected to indirect immunofluorescence analysis using indicated antibodies and were subsequently analyzed by CLSM. Inset images show enlargements of representative areas of the nuclear lamina. Filled arrowheads indicate accumulation of PKCα-GFP at the nuclear rim.
two N-terminally deleted constructs was capable of altering the nuclear distribution of pUL53 (Fig. 15A, panels i-q; compared to pUL53 alone in panels a-d). This result confirmed the mapped interaction domain comprising amino acids 1-181 of pUL50 assuming that binding of pUL50 to pUL53 is a prerequisite for the recruitment of pUL53. Furthermore, these two N-terminally deleted pUL50 constructs were also investigated in regard to their ability to recruit PKCα (Fig. 15C). In the resulting confocal images, the recruitment of PKCα by pUL50 was not as strong developed as observed by wide-field microscopy (Fig. 9). However, coexpression with full-length pUL50 or pUL50(20-397), respectively, resulted in a slight accumulation of PKCα at the nuclear rim (Fig. 15C, panels f-p), whereas N-terminal deletions up to amino acid 150 did not show any recruitment of PKCα (Fig. 15C, panels q-u). Thus, only the pUL50 variant which possessed the identified interaction region (i.e. amino acids 100-280) was able to recruit PKCα towards the nuclear rim. These data supported the CoIP results confirming the mapped interaction domains. In addition, these results indicate that recruitment of pUL53 and PKCα is directly dependent on the interaction with pUL50.

E-1.7.4 Importance of single amino acids in the N-terminus of pUL50 for binding pUL53

Previous experiments demonstrated that the N-terminal 15 residues of pUL50 were important for binding pUL53, with amino acids 10-14 being absolutely essential (Fig. 14). Furthermore, this region was predicted to be part of an α-helix, which is probably not part of the globular domain itself but rather forms an independent structural element (Fig. 12). Closer inspection of side chain properties disclosed the segregation of hydrophobic and polar residues between the two opposite faces of this α-helix (Fig. 16A and B), which is characteristic for amphipathic helices (Drin and Antonny, 2010). In order to further analyze the importance of single residues for binding pUL53, pUL50 expression constructs were cloned containing individual amino acid exchanges to alanine in the essential region comprising amino acids 10-14. Two pUL50 mutants with single amino acid exchanges on the hydrophilic/polar side (i.e. D10A and Q13A) and one double-mutant with two exchanges on the hydrophobic side (i.e. L11A/V12A) were generated (Fig. 16B). Consequently, these mutants were used for CoIP analysis to determine their ability to interact with pUL53 (Fig. 16C). In the case of the two single mutants, amino acid exchanges in the polar side of the amphipathic helix were tolerable for interaction (Fig. 16C, upper panel, lanes 4 and 6), though the CoIP signal compared to full-length pUL50 (Fig. 16C, upper panel, lane 2) was weaker, especially for mutation D10A (Fig. 16C, upper panel, lane 4). However, no CoIP signal was detected in the case of the double mutant (Fig. 16C, upper panel, lane 5), indicating the loss of interaction with pUL53. Particularly noteworthy, immunostaining of the CoIP blot with a pUL50-specific antibody reveals that only low amounts of the double-mutant were precipitated (Fig. 16C, middle panel, lane 5), compared to the single mutants (Fig. 16C,
middle panel, lanes 4 and 6) or full-length pUL50 (Fig. 16C, middle panel, lane 2). In addition, especially for the double-mutant, high amounts of lower migrating bands were detected (Fig. 16C, middle panel, lanes 2-6), indicating high levels of degradation. Nevertheless, repetition of this experiment yielded the same result (data not shown) confirming the low stability of the pUL50 double-mutant (L11A/V12A) and the decrease of CoIP signal for binding pUL53 under the detection limit.

**FIGURE 16. Influence of amino acid exchanges in pUL50 on its ability to bind pUL53.** (A and B) Schematic presentation of an amphipathic α-helix formed by amino acids 1-21 of pUL50 based on the steric configuration of side chains. The model is depicted in lateral (A) and top view (B). Amino acids are illustrated in stick presentation and colored according to side chain properties as indicated. (B) Only amino acids were labeled which were chosen for generation of expression mutants with amino acid exchange to alanine. (C) HA-tagged full-length pUL50 or pUL50 mutants were transiently coexpressed in 293T cells with FLAG-tagged pUL53 as indicated. At 2 days post-transfection, CoIP analysis was performed using mAb-HA. Coimmunoprecipitates and expression control samples were subjected to Western blot analysis using tag-specific antibodies as indicated. Ig-HC/LC, cross-reactive band for immunoglobulin heavy/light chain.
E-1.8 Late accumulation of viral and cellular proteins at the nuclear envelope of HCMV-infected cells

Experiments described above suggested the formation of a multi-protein complex which is associated with the nuclear lamina. To support this assumption, the intracellular localization of pUL53, pUL97 and p32 was investigated at different time points of infection (Fig. 17). Endogenous p32 was discernible in the cytoplasm of uninfected cells (mock), with a stringy appearance very characteristic of mitochondria (Fig. 17A, a-f; Matthews and Russell, 1998). Notably, the N-terminal MLS should be cleaved off from the mature p32, which then should exert nucleo-cytoplasmic shuttling activity triggered by phosphorylation of p32 (Majumdar et al., 2002; Brokstad et al., 2001). On the one side, the failure to detect the nuclear portion of p32 in this assay may be due to the use of an antibody that fails to recognize phosphorylated p32. On the other side, detection of nuclear p32 may be increased by the use of other fixatives than paraformaldehyde (Brokstad et al., 2001). However, the characteristic mitochondrial pattern observed in uninfected cells disappeared and was replaced by a more compact p32 distribution in the cytoplasm beginning with 24 hpi (Fig. 17A, panels g-m). Later during infection, p32 accumulated in small speckles (48 hpi; Fig. 17A, panels n-s) and finally in larger aggregates (72 hpi; Fig. 17A, panels t-y). Higher magnification revealed a clear tendency of p32 to string at the nuclear rim of infected cells, especially at 72 hpi (Fig. 17A, compare panels x-y with e-f). As a marker of infection, pUL53 was used for costaining. Since pUL53 was not detectable at 24 hpi, infected cells were separated from uninfected cells in regard to the altered p32 localization (Fig. 17A, compare panels l-m and e-f). Beginning with 48 hpi, pUL53 was distributed in a speckled pattern at the nuclear rim (Fig. 17A, panels p and v), similar to the localization when pUL53 was coexpressed with pUL50 in transiently transfected cells (Fig. 3 and 4). Due to this fact and observations obtained from cells infected with HSV-1 (e.g. Reynolds et al., 2001) or MCMV (e.g. Muranyi et al., 2002), HCMV-encoded pUL50 is likely to colocalize with pUL53 in infected cells. Contrary to pUL53, pUL97 was observed at 24 hpi in small subnuclear structures (Fig. 17B, panels e-h). Beginning with 48 hpi, pUL97 was mostly located in viral replication compartments (Fig. 17B, panels i-q) as described previously (Marschall et al., 2003). With increasing time, a number of cells also contained pUL97 in a perinuclear localization, which was at least in part associated with the nuclear envelope (Fig. 17C, panel f). Notably, due to the lack of suitable antibodies for the detection of pUL50 and endogenous PKCα in immunofluorescence analyses, the respective immunostainings could not be performed. However, these data further confirm the formation of protein complexes by viral and cellular proteins at the nuclear envelope of HCMV-infected cells.
FIGURE 17. Intracellular localization of viral and cellular proteins during the time course of infection. HFFs were infected with HCMV strain AD169 at an MOI of 1.0, or remained uninfected (mock) as indicated. At 24 hpi to 72 hpi (A and B) or 60 hpi to 120 hpi (C), the cells were subjected to indirect immunofluorescence analysis using indicated antibodies. Samples were subsequently analyzed by CLSM.
E-2 **Morphological alterations of the nuclear lamina during HCMV replication**

E-2.1 **Reorganization of the nuclear lamina in transiently transfected cells**

E-2.1.1 Lamin A/C reorganization induced by pUL97 and PKCα

In the course of cytomegaloviral lytic infection, the phosphorylation-dependent disassembly of the nuclear lamina is believed to be a prerequisite for nuclear capsid egress. In this regard, the pUL50-mediated recruitment of protein kinases such as pUL97 and PKCα (see E-1) appeared to be important. Therefore, the protein kinases were analyzed by immunofluorescence analysis concerning their effect on the distribution of lamin A/C, as a major component of the nuclear lamina (Fig. 18). In vector-transfected cells, endogenous lamin A/C was uniformly localized at the nuclear rim (Fig. 18A, panels a-e). Strikingly, this rim staining decreased in its intensity, together with an increasing amount of lamin signals throughout the nuclei in cells expressing pUL97 (Fig. 18A, panels f-k). Further experiments with a catalytically inactive point mutant of pUL97 (K355M), as well as catalytically active and inactive truncation mutants of pUL97 illustrated that its protein kinase activity was responsible for induction of the observed lamin A/C alterations (data not shown; Milbradt et al., 2010). Importantly, coexpression of pUL97 with the nuclear egress protein pUL50 potentiated the lamina-reorganizing activity. This was manifested by punctate distortions occurring at the periphery of the nuclear envelope, which clearly represented centers of massive lamin A/C reorganization in a subset of pUL97/pUL50 expressing cells (Fig. 18A, panels q-u). The potentiating effect of pUL50 might be explained either by the increased pUL97 recruitment mediated by the interaction of pUL50 with p32 (in complex with pUL97), or by the additional recruitment of endogenous PKC or other cellular protein kinases by pUL50. In contrast, coexpression of pUL53 and pUL97 did not induce lamin A/C reorganization as presented for pUL50 and pUL97 (data not shown). Notably, individually expressed pUL50 induced minor lamin A/C alterations restricted to a small fraction of cells, with no local depletion of the nuclear lamina in any case (Fig. 18A, panels l-p). In addition, expression experiments with PKCα-GFP were performed to investigate whether PKCα can also induce nuclear lamina alterations. Similar to pUL97, massive lamin A/C reorganization was detected in PKCα-expressing cells as well, actually without pUL50 (Fig. 18A, panels v-z). Intriguingly, these morphological alterations were locally restricted to punctate distortions of the lamin A/C rim-structure (Fig. 18B). In contrast to lamin A/C, the nuclear rim localization of lamin B was not altered by overexpression of pUL50/pUL97 or PKCα (data not shown). This finding is consistent with reports of the nuclear lamina reorganization during mitosis, stating that
lamin B remains membrane-associated while lamin A/C transiently becomes soluble (Broers et al., 2006; Dechat et al., 2008).

**FIGURE 18. Induction of lamin A/C reorganization by pUL97 and PKC activity.** (A and B) HeLa cells were transfected individually with pcDNA3.1 (vector), constructs encoding tagged versions of pUL97, pUL50, PKCα or combinations as indicated. At 24 h post-transfection, cells were fixed and coimmunostained with tag-specific antibodies (pAb-HA, pAb-FLAG) and mAb-lamin A/C. Samples were subsequently analyzed by CLSM. (B) Image of lamin A/C taken from a movie generated from z-stacks of a PKCα-GFP overexpressing cell. Open arrowheads indicate regions of massive nuclear lamina reorganization.

**E-2.1.2 Quantification of kinase-dependent lamin A/C alterations**

Since the induction of massive lamin A/C reorganization was only observed in a subset of transfected cells, it had to be guaranteed that this was a specific effect of the overexpressed protein kinases. For confirmation, the number of cells which exhibit massive alterations of the
lamin A/C staining were compared to the number of cells overexpressing pUL97 (in combination with pUL50) or PKCα, respectively. In both cases, quantification revealed that the percentage of transfected cells exhibiting distortions of lamin A/C had a maximum at 12 h post-transfection and thereafter decreased over time (Fig. 19A). This result may either indicate that the observed phenotypical effect was reversible for that the rim staining of lamin A/C was reconstituted at later time points, or, that the cells possessing massive lamin A/C alterations underwent apoptosis or necrosis. In order to exclude kinase-independent effects, the catalytically inactive point mutant pUL97(K355M) was used and, in addition, treatment with the protein kinase inhibitor Gö6976 was performed (Fig. 19B). The indolocarbazole compound Gö6976 possesses strong inhibitory potential against pUL97 (Marschall et al., 2001, 2002) and an additional activity against PKC (Goekjian and Jirousek, 1999). Notably, quantification of pUL50/pUL97-coexpressing cells revealed that 8.2% of cells staining positive for pUL50 exhibited distinct distortions of lamin A/C (Fig. 19A and B). Treatment with Gö6976 reduced this effect to 1.8%, confirming the central role of protein kinase activity (Fig. 19B). Using pUL97(K355M) resulted in 2-fold decrease of lamin A/C distortions (4.4%) compared to wild-type pUL97 (Fig. 19B). The latter observation suggested that endogenous PKC activity, possibly associated with pUL97(K355M)-derived protein complexes, might also contribute to the alterations in lamin A/C. This notion was underscored by the finding that Gö6976 treatment markedly reduced the effect of pUL97(K355M) (2.6%; Fig. 19B).

**FIGURE 19.** Quantification of pUL97/pUL50- or PKCα-dependent lamin A/C alterations in transfected cells. (A) HeLa cells were cotransfected with constructs encoding tagged-versions of pUL97 and pUL50, or were transfected individually with PKCα-GFP. At 12 h to 72 h post-transfection, cells were subjected to immunofluorescence analysis. Percentage of cells exhibiting massive lamin A/C reorganization was evaluated among cells staining positive for pUL50 or PKCα, respectively. (B) Massive lamin A/C reorganization induced by coexpression of pUL50 with either catalytically active pUL97 or inactive pUL97(K355M) were analyzed in the presence or absence of Gö6976 at 12 h post-transfection similar as described in (A). Statistical significance was calculated by Student’s t-test compared to pUL97/pUL50 expressing cells without Gö6976 treatment. *, p = 0.05; **, p = 0.01; ***, p = 0.001. (A, B) >700 cells were scored in 5 to 10 independent evaluations in each case.
E-2.1.3 Analysis of lamin A/C alterations regarding the integrity of the nuclear envelope

Next, the question was addressed whether the massive distortions of lamin A/C may cause a dysfunction of the nuclear membrane permeability. Therefore, the localization of a large nuclear protein, i.e. HCMV immediate early (IE) protein IE2p86 in fusion with GFP (~115 kDa), was analyzed in transfected cells (Fig. 20). Interestingly, the massive morphological alterations of lamin A/C did not lead to any detectable nucleo-cytoplasmic diffusion of IE2p86-GFP (Fig. 20, panels l-p). In particular, an invariable nuclear localization was noted for IE2p86-GFP, and no difference was found when comparing cells coexpressing IE2p86-GFP with those expressing pUL50 (Fig. 20, panels a-e), pUL97 (panels f-k), pUL50 and pUL97 (panels l-p), or a vector control (data not shown). The integrity of the nuclear envelope was hence not been affected by the massive reorganization of lamin A/C indicating an exclusive destabilization of the nuclear lamina.

FIGURE 20. Massive lamin A/C reorganization without loss of integrity of the nuclear envelope. HeLa cells were cotransfected with combinations of constructs encoding pUL50-HA, pUL97-F and IE2p86-GFP as indicated. Cells were fixed at 12 h post-transfection, immunostained with mAb-lamin A/C and analyzed for nucleo-cytoplasmic shuttling of IE2p86-GFP by CLSM.

E-2.2 Induction of lamina-depleted areas by combined activities of pUL97 and PKC in HCMV-infected cells

The individual protein kinase activity of pUL97 or PKCα was sufficient to induce massive lamin A/C alterations in the setting of transient transfection. In order to analyze their combined effect, the reorganization of lamin A/C was investigated in the context of HCMV infection by immunofluorescence analysis (Fig. 21). First, in cells infected with HCMV strain AD169, association of pUL97 with replication compartments was used as a marker for the late phase of viral replication (Fig. 21A, panel f). Intriguingly, pronounced distortions of lamin A/C were only induced in HCMV-infected cells (Fig. 21A, panels e-h). These distortions of the regular rim
staining, where no lamin A/C was detected, are referred to as lamina-depleted areas in the following. No case of lamina-depleted areas was found in uninfected cells (Fig. 21A, panels a-d). When using an AD169-derived UL97-deletion virus expressing a GFP reporter, limited distortions of lamin A/C were observed (Fig. 21A, panels i-m). This type of distortion was characterized by a limited thinning of the lamin A/C rim staining and by smaller depletions appearing with lower quantity compared to parental HCMV strain AD169. Quantification of the data revealed that most of the lamin-specific effect could be attributed to the presence of pUL97 (Fig. 21B). The lack of viral pUL97 expression (AD169ΔUL97-GFP) and/or the inhibition of kinase activity by Gö6976 substantially reduced the effect of lamin A/C depletion. Thus, the measurable inhibitory potency of Gö6976 in cells infected with UL97-deletion virus pointed to the additional importance of PKC to this phenotype (Fig. 21A, panel n). However, due to Gö6976 did not result in that the regular rim staining of lamin A/C was retained in each of the infected cells (AD169 and AD169ΔUL97-GFP; Fig. 21B), an additional effect of other cellular protein kinases could not be excluded.

**FIGURE 21. Analysis of lamin A/C alterations in HCMV-infected cells by CLSM.** (A) HFFs remained uninfected (mock, panels a-d) or were infected with HCMV strain AD169 (panels e-h) or recombinant AD169ΔUL97-GFP (panels i-n) at an MOI of 1.0. A subset of cells was treated with Gö6976 24 h prior to immunofluorescence analysis (panel n). At 3 days post-infection (AD169) or 3 weeks post-infection (AD169ΔUL97-GFP), cells were fixed and immunostained with indicated antibodies. Open arrowheads indicate lamina-depleted areas. (B) Kinase-dependent lamin A/C alterations were quantified in HCMV-infected cells in presence or absence of Gö6976; ~100 cells were scored in each case.
E-2.3 Visualization of HCMV nuclear capsid egress

E-2.3.1 Nucleo-cytoplasmic trafficking of GFP-labeled viral capsids in living cells

After having demonstrated the kinase-dependent induction of lamina-depleted areas in HCMV-infected cells, the next step was to investigate whether viral capsids preferentially use these sites for nuclear egress. First, confocal live cell imaging was performed to visualize the HCMV nuclear capsid egress. Therefore, infection experiments were performed using HCMV TB40 UL32-EGFP, recombinantly expressing pUL32-EGFP (pp150-EGFP) as a reporter protein. The reporter protein pUL32-EGFP is functional as a tegument protein and associates with viral capsids in the nucleus which allows tracking of viral capsids in living cells (Sampaio et al., 2005). A time-lapse experiment was started at 64 hpi and virus-producing cells were monitored over a period of 8 h. The use of direct live cell staining of the endoplasmic reticulum allowed the visualization of an individual fluorescent viral capsid trafficking from the internal area of the nucleus towards the nuclear envelope (Fig. 22, upper panels). Particularly noteworthy, transition of viral particles through the nuclear envelope could be detected (Fig. 22, lower panels). When examining the trafficking velocity of intranuclear viral capsids, a mean of 0.3-0.4 µm/s was determined (mean of individual capsids measured for 1.3-1.6 s over a distance of ~500 nm). Slightly different velocities were noted for nuclear compared to cytoplasmic trafficking of capsids (0.7-0.8 µm/s) The faster cytoplasmic movement was consistent with data published by others (Sampaio et al., 2005). Taken together, using GFP-labeled HCMV has been proven to be a reliable tool to visualize the nuclear capsid egress in living cells.

![Time-lapse series illustrating the nuclear egress of a GFP-labeled capsid at 3 days post-infection.](image)

FIGURE 22. Time-lapse series illustrating the nuclear egress of a GFP-labeled capsid at 3 days post-infection. HFFs were infected with HCMV TB40 UL32-EGFP at an MOI of 1.0. Live cell staining of the endoplasmic reticulum (red) preceded the examination of fluorescence by CLSM. The presented images are selected frames of a movie published as supplemental material of Milbradt et al., 2010. Filled arrowheads indicate the current position of the viral capsid; dotted lines indicate the trafficking of the viral capsid; Nu, nucleus; Cy, cytoplasm.

E-2.3.2 High resolution imaging of viral capsids adjacent to lamina-depleted areas

Next, HCMV TB40 UL32-EGFP was used to illustrate capsids during the viral replication cycle, particularly in regard to their localization compared to the nuclear lamina in immunostained fixed
cells. Entry of GFP-labeled particles was detected directly after the experimental adsorption phase (0 hpi; Fig. 23A, panels a-e). Moreover, replication kinetics indicated that newly synthesized pUL32-EGFP was present mainly in the cytoplasm in an even distribution early during infection (24-48 hpi; Fig. 23A, panel g), whereas at later time points (starting with 72 hpi), pUL32-EGFP appeared in a capsid-associated, speckled pattern in both the nucleus and the cytoplasm (Fig. 23A, panels m, r and w). Particularly noteworthy, viral capsids accumulated at

![Diagram of tracking of GFP-labeled viral capsids throughout the replication cycle of HCMV. HFFs were infected with HCMV TB40 UL32-EGFP at an MOI of 1.0. Cells were fixed at various time points post-infection and immunostained with mAb-lamin A/C.](image)

**FIGURE 23.** Tracking of GFP-labeled viral capsids throughout the replication cycle of HCMV. HFFs were infected with HCMV TB40 UL32-EGFP at an MOI of 1.0. Cells were fixed at various time points post-infection and immunostained with mAb-lamin A/C. (A) Monitoring of GFP-labeled capsids during the time course of infection. (B) High resolution imaging of the nuclear egress of viral capsids (z stacks of two examples of HCMV-infected cells; panels f and g). **Filled arrowheads** indicate viral capsids; **open arrowheads** indicate lamina-depleted-areas.
the nuclear lamina starting at 72 hpi, and moreover, lamina-depleted areas were induced concurrently (Fig. 23A, panels p, u and z). Investigating corresponding regions by high magnification, viral capsids were found in close proximity to lamina-depleted areas (Fig. 23B, panels a-e). Furthermore, selected z stacks of those regions indicated viral capsids passing through lamina-depleted areas (Fig 23B, panels f and g). Thus, the data presented in Fig. 23 suggests an important role of lamina-depleted areas for the nuclear egress of HCMV capsids.

E-2.3.3 Effect of the protein kinase inhibitor Gö6976 on the viral nuclear capsid egress

Since inhibition of viral pUL97 and cellular PKC resulted in the decrease of lamina-depleted areas in HCMV-infected cells (Fig. 21), further infection experiments were performed to elucidate the effect of reduced nuclear lamina reorganization on the viral nuclear capsid egress. Therefore, in HCMV-infected cells treated with or without protein kinase inhibitors, the proportion of cells which possesses an accumulation of viral capsids in the cytoplasm was determined as an indicator of efficient HCMV nuclear egress (Fig. 24). At late time points, viral capsids accumulated in the cytoplasm in almost half of infected cells (43.7%). Inhibition of kinase activity by Gö6976 resulted in 2- to 4fold reduction of exported viral capsids to the cytoplasm (2 µM Gö6976: 11.9%; 0.5 µM Gö6976: 23.8%). In control settings, treatment with the tyrosine kinase inhibitor AG490 did not significantly influence the transport of viral capsids to the cytoplasm (35.7%). Hence, the direct relationship between less reorganization of the nuclear lamina and decrease of HCMV nuclear capsid egress supports the assumption of viral capsids leaving the nucleus by passing through lamina-depleted areas.

FIGURE 24. Reduced efficiency of HCMV nuclear capsid egress by treatment with Gö6976. HFFs were infected with HCMV TB40 UL32-EGFP at an MOI of 1.0. Cells were treated with different concentrations of Gö6976 and AG490 as indicated. Fluorescence microscopy was applied at 72 hpi to quantify infected cells providing accumulation or no accumulation of capsids in the cytoplasm. >300 cells were scored in 5 to 7 independent evaluations in each case. Statistical significance was calculated by Student’s t-test compared to infected cells without inhibitor treatment. **, p = 0.01; ***, p = 0.001; n.s., not significant.
E-3 Evidence for a novel molecular mechanism responsible for the HCMV-induced nuclear lamina disassembly

E-3.1 Phosphorylation-dependent generation of a putative Pin1-binding site at serine 22 of lamin A/C

Site-specific phosphorylation of nuclear lamins is generally accepted to be responsible for nuclear lamina disassembly during mitosis. The presented results in E-2 and data published by others suggest similar events during the nuclear capsid egress of HCMV and other herpesviruses (reviewed in Mettenleiter et al., 2009). In particular, pUL97-dependent phosphorylation of lamin A/C on the serine at residue 22 (Ser22) was suggested to interfere with head-to-tail interactions between lamins as a trigger for nuclear lamina disassembly (Hamirally et al., 2009). However, a detailed molecular mechanism was not proposed so far. In the search for a possible molecular mechanism for the nuclear lamina disassembly during HCMV infection, bioinformatical analyses were performed concerning the phosphorylation at Ser22 of lamin A/C (summary depicted in Fig. 25A). Searching the ELM database for known interaction motifs revealed that phosphorylated Ser22 is part of a phosphoserine-proline motif that is known to mediate interaction with a subset of WW domains (i.e. small structurally conserved adaptor domains). In general, WW domains differ considerably in their ligand-binding preferences: group I, II, and III WW domains recognize unphosphorylated proline-rich sequence motifs (e.g. PPXY, PPLP, and PPR); group IV WW domains recognize phosphorylated serine and threonine residues (Verdecia et al., 2000). In order to identify WW domain-containing proteins that represent potential interaction partners of nuclear lamins, only nuclear proteins were taken into account. Search in the UniProt database yielded a total of 4386 human nuclear proteins, of which 14 contain WW domains. In a next step, the 25 WW domains present in these 14 proteins were analyzed with respect to their ligand-binding properties. A multiple sequence alignment of these 25 WW domains demonstrated a unique arginine residue (Arg17) in the sequence of the peptidyl-prolyl cis/trans isomerase (PPIase) Pin1 (data not shown; details of the bioinformatics approach are presented in Milbradt et al., 2010). Interestingly, a basic residue, such as arginine, at the respective sequence position of the WW domain is a prerequisite for the recognition of phosphorylated ligands by group IV WW domains (Ng et al., 2008). However, Pin1 is a nuclear PPIase that binds phospho-serine (pSer) or phospho-threonine (pThr) followed by a proline, and is known to be essential for cell cycle progression (Zarrinpar and Lim, 2000; Sudol et al., 2001). Additional evidence for lamin binding by Pin1 is provided by the striking similarity of lamins with known high affinity Pin1 ligands (Verdecia et al., 2000). One of these ligands, which originates from the C-terminal domain of RNA polymerase II, shares sequence similarity with the N-terminus of lamin A (RNA polymerase II: SPTpSPS; lamin A: TPLpSPT).
Modeling the respective Pin1-lamin A interaction complex (Fig. 25B) illustrated that the phosphoryl group of the phospho-serine (pSer22) in lamin A favorably interacts with a conserved serine (Ser16) and arginine (Arg17) of a Pin loop (Fig. 25C). None of these interactions can be formed by the unphosphorylated serine (Ser22; Fig. 25D). Particularly noteworthy, the potential Pin1 binding-site (TPLpSPT) can be found in A- and B-type lamins, indicating that phosphorylation of nuclear lamins at the respective serines generally creates a Pin1-binding site.

FIGURE 25. Discovery of a putative binding-site for cellular Pin1 at phosphorylated Ser22 of lamin A by bioinformatical analyses. (A) Flowchart for the prediction of Pin1 as an interaction partner of phosphorylated nuclear lamins. HMM, hidden markov model (B) Model of Pin1-lamin A interaction. WW domain of Pin1 is depicted in surface presentation and colored according to electrostatic potential (red, most negative; blue, most positive). Ser18 to Thr24 of lamin A are displayed in stick presentation and colored accordingly to the atom type. The boxed region is shown as an enlargement below. (C) Detailed view of the interactions formed by phosphorylated Ser22 (pSer22). The phosphoryl group forms tight interactions with Ser16 and Arg17 of Pin1 (yellow dotted lines). (D) The respective interactions cannot be formed by unphosphorylated Ser22.
E-3.2 Direct interaction of Pin1 with lamin A in HCMV-infected cells

In order to experimentally confirm the in silico approach, the postulated Pin1 interaction with lamin A was addressed by CoIP analysis of HCMV-infected cells. Therefore, cells were harvested during the late phase of viral replication (72 hpi), and subjected to CoIP with a Pin1-specific antibody. Lamin A was coimmunoprecipitated with Pin1 under low and high MOI conditions (Fig. 26A, lanes 2-3), contrary to uninfected cells, where the signal remained below the detection limit (Fig. 26A, lane 1). A control with preimmune serum illustrated the specificity of the reaction with only marginal background (Fig. 26A, lane 4). Moreover, relative signal intensities were quantified by densitometry indicating a significant Pin1-specific CoIP of lamin A (15.1fold/11.2fold increase versus background), whereas lamin C bound to Pin1 with much lower affinity (1.9fold/1.7fold). The additional staining patterns on precipitation and expression control Western blots confirmed the Pin1 specificity of the CoIP antibody and the reliable expression of proteins (Fig. 26B).

FIGURE 26. Interaction of Pin1 with lamin A in HCMV-infected cells. HFFs were infected with HCMV strain AD169 at an MOI of 0.1 to 1.0, or remained uninfected (mock). At 3 days post-infection, cells were lysed and used for CoIP analysis with pAb-Pin1 (A302-315A) or preimmune serum (pre) as indicated. Detection of coimmunoprecipitates (A) or precipitation and expression controls (B) was performed on Western blots using mAb-lamin A/C or pAb-Pin1 (A302-316A), respectively. Relative intensities of CoIP signals versus mock/preimmune serum conditions were determined by densitometry.

E-3.3 Effect of HCMV-infection on the intracellular localization of Pin1

Identifying the Pin1 interaction with lamin A by CoIP analysis suggested that Pin1 PPlase activity might play a role in the nuclear lamina disassembly during HCMV infection. The assumption that Pin1 is involved in the nuclear lamina disassembly was analyzed by confocal imaging.
concerning the intracellular localization of Pin1 in HCMV-infected cells. In addition to staining Pin1, costaining of lamin A/C and of the viral DNA polymerase processivity subunit pUL44 was performed to obtain markers for the localization of the nuclear lamina and viral replication compartments, respectively (Fig. 27 and 28). In uninfected cells, Pin1 was predominantly localized diffusely throughout the nucleus slightly accumulating at the nuclear rim (Fig. 27, panels a-d). Starting with 24 hpi, the Pin1 distribution altered towards a distinct accumulation in viral replication compartments (Fig. 27, panels e-m). This early recruitment of Pin1 was unexpected, since the postulated involvement of Pin1 in the HCMV nuclear capsid egress suggests an implication in events later during infection. Thus, its functional relevance remains to be elucidated in future studies.

FIGURE 27. Subnuclear relocalization of Pin1 early during infection. HFFs were infected with HCMV strain AD169 or remained uninfected (mock) as indicated. Cells were fixed at 24 hpi or 48 hpi for coimmunostaining with pAb-Pin1 (H-123), mAb-lamin A/C and mAb-UL44. Samples were analyzed by CLSM.

During the late phase of viral replication, strikingly, Pin1 was recruited not only to the periphery of viral replication compartments (Fig. 28A, panels a-f), but also partly to specific depletion sites of lamin A/C (Fig. 28A, panels g-m). Concerning the specificity of the Pin1 recruitment, individually stained preparations were performed in parallel with one antibody at the same time to exclude cross-talk of the used dyes (data not shown). Next, the cause of Pin1 recruitment was investigated in more detail. Since the induction of lamina-depleted areas during HCMV infection was dependent on the activity of pUL97 and PKC (Fig. 21), it was investigated whether protein kinase activity is also responsible for the recruitment of Pin1 to the nuclear lamina. For this purpose, Pin1 localization was visualized in HCMV-infected cells treated with protein kinase inhibitors. Treatment with the pUL97/PKC inhibitor Gö6976 reduced the formation of lamina-depleted areas and, importantly, Gö6976 reduced also the accumulation of Pin1 at the nuclear lamina (Fig. 28B, panels a-f). Notably, the tyrosine kinase inhibitor AG490 did not reduce the HCMV-induced Pin1 relocalization (Fig. 28B, panels g-m). These novel findings strongly
support the prediction of a phosphorylation-triggered generation of a Pin1-binding site within lamin A/C. Thus, the cellular PPIase Pin1 is a candidate for exerting a so far unrecognized mechanism of lamina reorganization in HCMV-infected fibroblasts.

FIGURE 28. Kinase-dependent relocalization of Pin1 late during infection. (A and B) HFFs were infected with HCMV strain AD169 or remained uninfected (mock) followed by coimmunostaining with pAb-Pin1 (H-123), mAb-lamin A/C and mAb-UL44 at 72 hpi as indicated. HCMV-infected cells were treated with 2 μM Gö6976 or 2 μM AG490 (B). Open arrowheads indicate lamina-depleted areas.
F Discussion

F-1 Formation of a viral-cellular nuclear egress complex (NEC)

This study was initiated in order to reveal the molecular events which are responsible for the nuclear egress of HCMV. Experimental results of previous studies suggested that the herpesviral homologs of the lamina-associated HCMV-encoded proteins pUL50 and pUL53 play a pivotal role for the translocation of viral capsids from the nucleus into the cytoplasm (Sanchez and Spector, 2002). In particular, reports using recombinant HSV-1, with deletion of either ORF UL34 or UL31, initially described the lack of enveloped virus particles in the cytoplasm of infected cells (Roller et al., 2000; Chang et al., 1997). Interaction of UL34 and UL31 has been demonstrated to result in the formation of a protein complex that accumulates at the nuclear membrane (Reynolds et al., 2001; Liang and Baines, 2005). Similar formation of UL34-UL31 complexes has been described for the homologous proteins of PrV, MCMV and EBV (Fuchs et al., 2002; Bubeck et al., 2004; Schnee et al., 2006; Muranyi et al., 2002; Lake and Hutt-Fletcher, 2004). Since interaction of these proteins has been shown to be required for the export of viral capsids from the nucleus, this complex was referred to as the nuclear egress complex (NEC) (Lötzerich et al., 2006; Mettenleiter et al., 2009).

In this work, NEC formation by HCMV-encoded pUL50 and pUL53 was investigated. Using immunofluorescence costaining analyses, pUL50 was shown to relocalize pUL53 from a diffuse nuclear localization to a distinct rim pattern associated with the nuclear envelope. Upon coexpression, both proteins colocalized with components of the nuclear lamina such as lamin A/C and LBR. Direct interaction between pUL50 and pUL53 was demonstrated in yeast two-hybrid and CoIP analyses, ultimately demonstrating that complex formation at the nuclear membrane between the nuclear egress proteins is also conserved in HCMV. This result confirmed a previous report in which HCMV pUL50 and pUL53 interacted in a protein complementation assay (Schnee et al., 2006), and coincided with a biochemical approach characterizing the interactions within the pUL50-pUL53 complex (Sam et al., 2009). Unlike the study of Sam et al. (2009), no evidence was found for interaction of pUL53 with itself in this work. Apparent discrepancies may be due to the use of pUL53 truncation mutants by Sam et al. (2009), which might lead to defective protein folding and possibly to the detection of a false positive interaction. Another explanation is provided by a possibly non-physiological protein folding of pUL53 in this study (yeast two-hybrid analysis presented in E-1.3), potentially resulting in a failure to form homodimers (putative homodimerization region mapping to amino acids 50-83; Sam et al., 2009). Another report suggested that the corresponding region in the MCMV homolog of pUL53 is required for binding the homolog of pUL50 (Lötzerich et al., 2006). This indicated that pUL50 might compete for an interaction surface on pUL53 that was required for
mediating pUL53 homodimerization. However, the affinity of pUL50 for pUL53 appeared to be stronger than the interaction of the pUL53 homodimer, suggesting that pUL50-pUL53 heterodimerization was preferred (Sam et al., 2009). In regard to pUL50, mapping analysis revealed in this thesis, that a highly conserved region of pUL50, ranging from amino acids 1-181, was involved in the binding of pUL53. This observation is in line with a report demonstrating that a truncation mutant encoding amino acids 1-169 of pUL50 was able to interact with pUL53 (Sam et al., 2009). In this study, the amphipathic α-helix in the N-terminus of pUL50 emerged to be particularly important for binding pUL53. N-terminal truncation mutants of pUL50 resulted in the gradual decrease of binding affinity, while deletion of amino acids 1-14 resulted in the complete loss of interaction with pUL53. Moreover, individual amino acid exchanges of residues on the hydrophilic side of the amphipathic α-helix induced only a decrease of binding affinity but not the loss of interaction. Notably, a pUL50 double-mutant which contains two mutations in the hydrophobic side of the α-helix showed increased degradation and loss of protein stability as demonstrated by Western blot analysis. This suggests that distinct residues on the hydrophobic side may anchor the α-helix into the globular domain to sustain pUL50 stability. In addition, no interaction with pUL53 was measurable for the remaining detectable portion of this pUL50 double-mutant. Thus, these data together indicate that a combined interface within pUL50, consisting of the hydrophilic side of an N-terminal α-helix and parts of the globular domain, is involved in binding pUL53.

Interestingly, further direct interactions of pUL50 with p32 and PKC suggested a more complex situation than the sole formation of a pUL50-pUL53 heterodimer in HCMV-infected cells. Despite the identification of partly overlapping interaction domains for its three interaction partners, pUL50-associated protein complexes were detected in transiently transfected and HCMV-infected cells. This favors the idea of simultaneous binding of at least two proteins (i.e. pUL53 and PKC) to pUL50. Particularly noteworthy, in addition to the interaction with pUL50, p32 is known to bind various proteins including pUL97, PKC isoforms and LBR (Marschall et al., 2005; Storz et al., 2000; Robles-Flores et al., 2002; Mylonis et al., 2004). Whether these interactions occur simultaneously or in a temporal order of events has still to be determined. Finally, the finding that these proteins accumulate at the nuclear envelope upon coexpression in transiently transfected cells and during the late phase of viral replication, leads to the hypothesis that the NEC consists of at least six proteins (i.e. pUL50, pUL53, p32, LBR, PKC and pUL97) (Fig. 11). In particular, these data suggest that the cellular adaptor protein p32 might act as the central component of the NEC. Along with the transmembrane protein pUL50, p32 might establish the direct association of the NEC with the nuclear lamina by interacting with the LBR. Further as yet unidentified proteins and protein kinases may additionally be involved. Especially a putative cellular protein mediating the association of pUL53 with p32 might provide
an explanation for some contradictory results from CoIP and yeast two-hybrid analyses in regard to detectable/non-detectable interaction between pUL53 and p32, respectively.

**F-2 Important roles of nuclear egress proteins pUL50 and pUL53**

To which extent HCMV pUL50 and pUL53 as well as their homologs contribute to nuclear egress has been investigated for several years especially in α-herpesviruses (Reynolds et al., 2004; Bjerke and Roller, 2006; Mou et al., 2009; Camozzi et al., 2008; Milbradt et al., 2007). In this study, individual overexpression of pUL50 only induced minor alterations of lamin A/C in a subset of cells. Similarly, coexpression of pUL50 with pUL53, in the absence of any other viral protein, had also marginal effects on lamina A/C. In a report by Camozzi et al. (2008), results were presented indicating that pUL50 and pUL53 have a more drastic effect on the nuclear lamina of transiently transfected cells (Camozzi et al., 2008). However, no quantitative determination was given from which one could conclude the percentage of cells showing this phenotype. For the HSV-1 homologs, it was suggested that UL34 and UL31 might directly modify the confirmation of the nuclear lamina by a direct interaction with lamin A/C (Reynolds et al., 2004). However, the direct protein-protein interaction between nuclear lamins and HCMV pUL50 and pUL53 has not been demonstrated so far.

As an important point, several reports suggested that pUL50-/pUL53-associated or UL34-/UL31-associated protein kinases are likely to be involved in the nuclear egress (Bjerke and Roller, 2006; Park and Baines, 2006; Marschall et al., 2005). Here, evidence is provided that overexpression of viral pUL97 or cellular PKC is sufficient to induce a specific type of reorganization of lamin A/C. Interestingly, pUL50 was able to recruit both protein kinases, pUL97 and PKC, to the nuclear lamina. Contrary to the pUL53-independent recruitment of PKC by pUL50, HSV-1 UL34 and UL31 are both required for PKC recruitment to the nuclear lamina in HSV-1-infected cells (Park and Baines, 2006). In the present study, a specific situation was described for the association of pUL97 with pUL50 and the pUL50-associated complex. Whereas PKC recruitment was mediated by interaction with pUL50, pUL97 was recruited through a different mechanism lacking direct interaction. It had previously been demonstrated that cellular p32 recruits pUL97 to the nuclear lamina by association with the LBR (Marschall et al., 2005). Against this background, the observed interaction between p32 and pUL50 was thought to direct the pUL97-p32 complex to the nuclear lamina. In summary, the proper formation and localization of the pUL50-pUL53 complex is highly suggestive to be required for the regulation of nuclear lamina disassembly by recruiting pUL97 and/or PKC to specific target sites of nuclear capsid egress.

The ability of pUL50 and pUL53 to induce the reorganization of the nuclear lamina by direct or indirect means suggests that the postulated holistic NEC may not permanently be required for
nuclear lamina disassembly. An intriguing possibility is that NEC formation might be temporally coordinated in a sequence of interaction processes which may be dependent on specific trigger events. Evidence for this hypothesis is provided by observations in HSV-1-infected cells. In particular, HSV-1-encoded US3 and UL13 kinases are believed to mediate the initial disassembly of the nuclear lamina (Lee and Chen, 2010). Thereafter, US3 regulates the formation and proper localization of the UL34-UL31 complex at the nuclear lamina by phosphorylation of the two proteins (Kato et al., 2006; Reynolds et al., 2002; Ryckman and Roller, 2004). Due to the direct interaction with lamin A/C, further reorganization of the nuclear lamina may then be induced by UL34 and UL31 (Reynolds et al., 2004). In addition, PKC is simultaneously recruited to the nuclear lamina by the UL34-UL31 complex, and PKC activity finally appears to lead to the integral disassembly of the nuclear lamina during nuclear egress of HSV-1 (Leach et al., 2007; Park and Baines; 2006; Lee and Chen, 2010). In the case of HCMV, primary rearrangement of the nuclear lamina may be effected or at least facilitated by activity of the virion-associated part of pUL97, due to the fact that very early phosphorylation (i.e. 4 hpi) of lamin A/C has been described (Buchkovitch et al., 2010). In the present work, it was demonstrated that phosphorylation of pUL50 is mediated by PKC. pUL50 phosphorylation might be one of the regulatory steps required for the physiological association of the pUL50-pUL53 complex and targeting to the nuclear lamina in HCMV-infected cells. Consequently, pUL50 and pUL53 might then induce the formation of an enlarged entity of the NEC, particularly by the recruitment of protein kinases, such as pUL97 and PKC. In the later steps of this process, lamin phosphorylation might both trigger conformational rearrangement as well as restriction of the resulting lamina depletion to certain areas (thus assuring the absence of a complete breakdown of the nuclear envelope).

In addition to functions required for the rearrangement of the nuclear lamina, pUL50 and pUL53 may be also involved in terminal steps of nuclear egress. For HCMV, the processes required for primary envelopment and subsequent deenvelopment of viral capsids still remain to be defined. However, for HSV-1, there is evidence that viral glycoproteins are present in primary enveloped virions (Farnsworth et al., 2007). This suggests that HSV-1 glycoproteins accumulate at primary budding sites at the INM prior to or at the time of envelopment (Wills et al., 2009). Interestingly, HSV-1 UL34 and UL31 were demonstrated to be involved in recruiting glycoproteins to these budding sites (Wills et al., 2009). Since HSV-1 glycoproteins promote fusion between the primary envelope and ONM (Wisner et al., 2009), the direct or indirect involvement of HSV-1 UL34 and UL31 in the deenvelopment of viral capsids during nuclear egress has been discussed (Lee and Chen, 2010). Interestingly in case of HCMV infection, a direct interaction of pUL50 with the ER chaperone BiP was described. A regulatory role of this interaction for nuclear lamina disassembly, or processes following nuclear egress required for maturation in the cytoplasm seem to be possible (Buchkovitch et al., 2010). Thus, functions of
HCMV pUL50 and pUL53 might reach beyond the described activities leading to the disassembly of the nuclear lamina and may include further steps of egress and maturation.

**F-3 Dependency of NEC function on protein kinases and further effectors**

Functionality of the postulated HCMV-specific NEC is not only based on the nuclear egress proteins pUL50 and pUL53, but also on further viral and cellular components. Here, novel experimental data are provided for the NEC-mediated reorganization of the nuclear lamina. Based on transient transfection experiments, infection with recombinant HCMVs and the use of protein kinase inhibitors, disassembly of the nuclear lamina could be attributed to viral and cellular protein kinases. Studies performed by our research group demonstrated the phosphorylation of transiently expressed versions and endogenous lamin A/C and B by pUL97 (Milbradt et al., 2010; Marschall et al., 2005). Hamirally et al. (2009) similarly described the direct pUL97-mediated phosphorylation of lamin A by the use of purified proteins. In both cases, the pUL97-mediated phosphorylation of nuclear lamins could be correlated to the lamina-reorganizing potential of pUL97. This was mainly concluded from the finding that catalytically active pUL97 was sufficient to induce morphological alterations of the nuclear lamina while catalytically inactive pUL97 was not (Hamirally et al., 2009; Milbradt et al., 2010; Marschall et al., 2005). In addition, cellular PKC activity was also found to be involved in HMCV-induced disassembly of the nuclear lamina. Supporting this, in HSV-1-infected cells, PKC isoforms α and δ are very efficiently recruited to the nuclear lamina (Park and Baines, 2006). Notably, the proapoptotic PKCδ is able to phosphorylate lamin B in vitro, which is a prerequisite for efficient nuclear lamina disassembly during apoptosis (Cross et al., 2000). Thus, along with pUL97, several isoforms of PKC appear to be involved in NEC function and, consequently, in the nuclear egress of herpesviruses.

An important hallmark of lamin phosphorylation in HCMV-infected cells is the site-specific phosphorylation of lamin A/C by pUL97 at serine 22. Serine 22 is also a phosphorylation site of CDK1 (Heald and McKeon, 1990; Peter et al., 1991). The CDK1 phosphorylation sites of the lamin types are part of conserved sequence stretches. For lamin B, the respective phosphorylated residues are serine 23 (lamin B1) and serine 17 (lamin B2) (Isobe et al., 2007; Peter et al., 1991). Lamin A and C are subject to alternative splicing of the LMNA gene and share the same N-terminal sequence including the CDK1/pUL97 phosphorylation site. In this study, first evidence is provided that phosphorylation of serine 22 of lamin A/C might generate a sequence pattern (TPLpSPT) which fulfills the minimal binding motif for the PPIase Pin1 (pSP) and, moreover, shares similarities to the respective sequence stretches of known ligands of Pin1.
It was highly encouraging to detect a Pin1-lamin A interaction and a relocalization of Pin1 in HCMV-infected fibroblasts which appeared to be dependent on protein kinase activity (pUL97 and/or PKC). The latter conclusion was based on the finding that protein kinase inhibitor Gö6976 strongly reduced Pin1 relocalization. The presented data describe the early recruitment of Pin1 to viral replication centers and a later partial recruitment to the nuclear envelope suggesting a novel scenario for the mechanism of nuclear lamina disassembly during HCMV nuclear egress. This mechanism might be explained by a Pin1-mediated modulation of the lamin head domains, possibly resulting in a decreased potency to form head-to-tail polymers. Particularly noteworthy, interference of head-to-tail interactions between nuclear lamins is believed to be a trigger for nuclear lamina disassembly (Hamirally et al., 2009; Kapinos et al., 2010). PPIases, such as Pin1, catalyze the cis/trans isomerization of peptidyl-prolyl peptide bonds which mediates conformational changes of proteins leading to altered protein folding (Fig. 29). Pin1-catalyzed switches regulate a spectrum of target activities comprising decrease and increase of protein stability or transcriptional activity, targeting of the subcellular protein localization and many more (Lippens et al., 2007; Lu et al., 2007; Lu and Zhou, 2007). An alternative, indirect mechanism may involve so far unknown effector proteins to bind to lamins as a consequence of Pin1-induced conformational changes. Such effector binding could eventually provide the stimulus for another molecular mechanism leading to disassembly of the nuclear lamina.

FIGURE 29. Conformational switch of pSer/Thr-Pro motifs induced by Pin1. Phospho-dependent cis/trans isomerization of proline residues is catalyzed by PPIase activity of Pin1. The resulting conformational switch is a determinant for protein function (modified from Lu and Zhou, 2007).

The presented CLSM data of the NEC-mediated reorganization of the nuclear lamina provides novel insights into HCMV nuclear egress. Various forms of distortions of the nuclear lamina were detected in HCMV-infected fibroblasts including the occurrence of massive lamin A/C reorganization (lamina-depleted areas). It should be mentioned that previous studies already reported effects of nuclear lamina disassembly during HCMV replication.
(Marschall et al., 2005; Camozzi et al., 2008; Hamirally et al., 2009) which remained, however, poorly characterized so far. This study describes for the first time the HCMV-specific induction of distinct, punctate lamina-depleted areas. This observation is strongly reminiscent of the infoldings of the INM free of lamina, described by Buser et al. (2007) by transmission electron microscopy. In their study, these infoldings were postulated as preferred sites where HCMV and MCMV capsids acquire their primary envelope by budding into the perinuclear space (Buser et al., 2007). The CLSM data presented in this work demonstrating the morphological alteration of the nuclear lamina in HCMV-infected cells, also including live-cell imaging of freely moving viral particles, strongly support the conclusion of the report by Buser and colleagues. Thus, the lamina-depleted areas may represent preferred sites of HCMV nuclear capsid egress.

In conclusion, the currently available data suggest that the activity of the postulated HCMV-specific NEC might trigger a novel scenario of nuclear lamina reorganization during infection (Fig. 30). In particular, this study indicates that NEC formation is responsible for the recruitment of viral and cellular protein kinases to the nuclear lamina (Fig. 30, step 1 and 2). The recruited protein kinases then might induce site-specific phosphorylation of nuclear lamins which generates a binding motif for the putative downstream effector Pin1 (Fig. 30, step 3). Importantly, combination of protein-protein interaction analyses with immunofluorescence data suggests that Pin1 is recruited to the nuclear lamina and binds directly nuclear lamins (Fig. 30, step 4). Pin1 recruitment may locally provide increased PPIase activity to execute a reorganization of the nuclear lamina (Fig. 30, step 5). Finally, this might result in the generation of lamina-depleted areas which are actively used for nuclear egress (Fig. 30, step 6). Further investigation will be necessary to gain a deeper insight into the specific molecular steps regulating this mechanism. Pin1 recruitment might be a conserved mode of phosphorylation-triggered reorganization of the nuclear lamina during the replication of HCMV and other herpesviruses.
**FIGURE 30.** Hypothetical model illustrating the course of events triggering the formation of lamina-depleted areas in HCMV-infected cells. See text for detailed description of individual steps. (P), substrate protein phosphorylation; phos., phosphorylation.
Abbreviations

aa  amino acid
AD  activation domain
BD  binding domain
CDK  cyclin-dependent kinase
CLSM  confocal laser-scanning microscopy
CoIP  coimmunoprecipitation
CPE  cytopathic effect
CR  conserved region
DAPI  4',6-diamidino-2-phenylindole
DMEM  Dulbecco's modified Eagle medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
E  early
EBV  Epstein-Barr virus
ECL  enhanced chemiluminescence
EDTA  ethylenediaminetetraacetic acid
eGFP  enhanced green fluorescent protein
ELM  eukaryotic linear motif
ER  endoplasmic reticulum
FCS  fetal calf serum
h  hour(s)
H2B  histone 2B
HA  hemagglutinin
HBS  HEPES-buffered saline
HCMV  human cytomegalovirus
HEK  human embryonic kidney
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF  primary human foreskin fibroblasts
HHV  human herpesvirus
HIV  human immunodeficiency virus
hpi  hours post-infection
HRP  horseradish peroxidase
HSV  herpes simplex virus
IE  immediate early
INM  inner nuclear membrane
IVKA  in vitro kinase assay
kbp  kilo basepair
kDa  kilo Dalton
L  late
LB  Luria-Bertani
LBR  lamin B receptor
LiAc  lithium acetate
mAb  monoclonal antibody
MCMV  murine cytomegalovirus
MCS  multiple cloning site
MEM  Eagle's minimal essential medium
min  minute(s)
MLS  mitochondrial localization signal
MOI  multiplicity of infection
NCR  non-conserved region
NEC  nuclear egress complex
NLS  nuclear localization signal
<table>
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<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>ONM</td>
<td>outer nuclear membrane</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>pAb</td>
<td>polyclonal antibody</td>
</tr>
<tr>
<td>PBSo</td>
<td>phosphate-buffered saline without CaCl$_2$ and MgCl$_2$</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylenglycol</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PPlase</td>
<td>peptidyl-prolyl cis/trans isomerase</td>
</tr>
<tr>
<td>PrV</td>
<td>pseudorabies virus</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>Rt</td>
<td>room temperature</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>STP</td>
<td>staurosporine</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate-EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tween</td>
<td>polyethylene glycol sorbitan monolaurate</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>US</td>
<td>unique short</td>
</tr>
<tr>
<td>VZV</td>
<td>varizella-zoster virus</td>
</tr>
<tr>
<td>Xgal</td>
<td>5-bromo-4-chloro-3-indoly-$\beta$-D-galactopyranoside</td>
</tr>
<tr>
<td>YNB</td>
<td>Yeast Nitrogen Base</td>
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I Appendix

Own Publications


* corresponding author


Contributions to national and international conferences

1. 4th European Congress of Virology, Cernobbio, Italy, April 2010
   Cytomegaloviral nuclear capsid egress through lamina-depleted areas induced by viral and cellular protein kinase activity.
   (Poster and Oral presentation)

2. 1st Retreat of Integrated Research Training Group (SFB 796), Atzelsberg, Germany September 2009
   Combined protein kinase activities of cytomegaloviral pUL97 and cellular PKC create lamina-free areas as potential sites for viral nuclear egress.
   (Oral presentation)

3. Leica Symposium, Erlangen, Germany, September 2009
   Visualization of cytomegaloviral nuclear capsid egress by confocal imaging.
   (Oral presentation)

4. 34th International Herpesvirus Workshop (IHW 2009), Ithaca, USA, July 2009
   Combined protein kinase activities of cytomegaloviral pUL97 and cellular PKC create lamina-free areas as potential sites for viral nuclear egress.
   (Poster presentation)

5. 19th Annual Meeting of the Society for Virology (GfV), Leipzig, Germany, March 2009
   Activity of cytomegaloviral protein kinase pUL97 creates lamina-free areas as potential sites of nuclear egress.
   (Poster and Oral presentation)

6. 6th International Conference on HHV-6 & 7, Baltimore, USA, June 2008
   Sensitivity of HHV-6 and other human herpesviruses towards the pluripotent drug artesunate.
   (Oral presentation)
(7) 18th Annual Meeting of the Society for Virology (GfV), Heidelberg, Germany, March 2008
(Oral presentation)

(8) 3rd European Congress of Virology, Nuremberg, Germany, September 2007
Association of the cytomegaloviral proteins UL50 and UL53 with the nuclear envelope and specific recruitment of PKC.
(Poster presentation)

Awards

(1) HHV-6 Foundation Award – Top Abstract, 6th International Conference on HHV-6 & 7
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Prof. Dr. Robert Slany, Genetics
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