Cellular p32 Recruits Cytomegalovirus Kinase pUL97 to Redistribute the Nuclear Lamina*

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Replication of human cytomegalovirus is limited at the level of nucleocytoplasmic transport of viral capsids, a process that requires the disassembly of the nuclear lamina. Deletion of the protein kinase gene UL97 from the viral genome showed that the activity of pUL97 plays an important role for viral capsid egress. Here, we report that p32, a novel cellular interactor of the viral kinase pUL97, promotes the accumulation of pUL97 at the nuclear membrane by recruiting the p32-pUL97 complex to the lamin B receptor. Transfection of active pUL97, but not a catalytically inactive mutant, induced a redistribution of lamina components as demonstrated for recombinant lamin B receptor-green fluorescent protein and endogenous lamins A and C. Consistent with this, p32 itself and lamins were phosphorylated by pUL97. Importantly, overexpression of p32 in human cytomegalovirus-infected cells resulted in increased efficiency of viral replication and release of viral particles. Thus, it is highly suggestive that the cellular protein p32 recruits pUL97 to induce a dissolution of the nuclear lamina thereby facilitating the nuclear export of viral capsids.

The transport of macromolecules in eukaryotic cells is subject to a strict compartmentalization into nucleus and cytoplasm. Exchange reactions between the two compartments are mediated through the nuclear pore complex, and thus the integrity of the nuclear envelope, composed of membrane and lamina constituents, is crucial for intracellular transport pathways. The nuclear lamina, underlining the inner nuclear membrane, contains a variable number of lamin isoforms (which are members of the intermediate filament family of cytoskeletal proteins) and forms a rigid, proteinaceous meshwork. During infection with herpesviruses, the nuclear lamina represents a barrier that hampers the nucleocytoplasmic transport of viral capsids (1). Because of the large size of herpesviral capsids (~120 nm), which does not allow their direct cytoplasmic release through nuclear pores, the structural destabilization of the nuclear lamina is an important prerequisite of virus budding. Lamina destabilization requires site-specific phosphorylation of lamins and lamin-binding membrane proteins. Phosphorylation leads to lamin depolymerization and may also permit their release from lamin-binding membrane proteins, including the lamin B receptor (LBR)2 (2, 3). Protein kinase C and Cdc2 have been identified as kinases phosphorylating lamins during mitosis (3, 4). Interestingly, protein kinase C is involved in the dissolution of the nuclear lamina in cells infected with murine cytomegalovirus (5). In addition to cellular protein kinases, the activity of virus-encoded protein kinases has been suspected as an important additional critical factor for nuclear export of herpesviruses, such as herpes simplex virus type 1 (HSV-1) and pseudorabies virus (6, 7).

Concerning the replication of human cytomegalovirus (HCMV), which is a major human pathogenic herpesvirus, little information has been published on destabilization of the nuclear lamina. HCMV causes severe forms of systemic disease in immunosuppressed patients and prenatally infected children. The most frequently applied antiviral therapy, i.e. intravenous or oral administration of the nucleoside analog ganciclovir, is based on the specific ganciclovir-phosphorylating activity of the UL97-encoded viral kinase (pUL97) (8, 9). Interestingly, pUL97 does not phosphorylate naturally occurring nucleosides but is a protein kinase with specificity for serine/threonine residues (10). Although having been studied intensively for more than a decade, the role of pUL97 within the HCMV replication cycle is still not known in detail. The functional importance of pUL97 was illustrated by experimental deletion of the UL97 coding region from the viral genome: although replication-competent UL97-deleted HCMV could be generated, replication of this virus is restricted to very low titers, indicating that pUL97 kinase activity is required for high efficiency of viral replication (11). This is also supported by the recent development of pUL97-specific protein kinase inhibitors with strong anti-cytomegaloviral effects, demonstrating that the kinase activity of pUL97 constitutes a novel attractive target for antiviral therapy (12, 13). Several studies on UL97-deleted virus mutants revealed that their low level replication is the result of defects in DNA synthesis and, even more pronounced, in nuclear export of viral capsids (14, 15).

To gain insight into the key role of pUL97 in nuclear capsid export, its interaction with cellular proteins was the focus of interest. Consequently, we performed a yeast two-hybrid screening using a human cDNA library and identified a novel cellular interactor of pUL97. Interestingly, this interactor, p32, is associated with a number of proteins, among them a component of the nuclear lamina, the LBR. Our data strongly suggest that p32 recruits the pUL97 kinase activity to this cellular compartment. Moreover, we could demonstrate a pUL97-mediated remodeling of the subnuclear structure of the lamina and a specific phosphorylation of lamins. This newly identified activity of pUL97 suggests a specific mechanism through which lamin destabilization and viral capsid export is promoted.

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2 The abbreviations used are: LBR, lamin B receptor; CoIP, communoprecipitation; CREB, cAMP response element-binding protein; dpi, days postinfection; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; H2B, histone 2B; HA, hemagglutinin; HCMV, human cytomegalovirus; HFF, human foreskin fibroblast; HIV-1, human immunodeficiency virus type 1; HSV-1, herpes simplex virus type 1; mAb, monoclonal antibody; MCP, major capsid protein; m.o.i., multiplicity of infection; TRITC, tetramethylrhodamine isothiocyanate. Superscript F and HA indicate FLAG-tagged and HA-tagged, respectively.
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EXPERIMENTAL PROCEDURES

Cell Culture and Viruses—Primary human foreskin fibroblasts (HFFs) were cultivated in minimum Eagle’s medium; 293, HeLa, and U373 cells in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum. HCMVs AD169 and AD169-GFP were propagated in HFFs, and virus replication was quantified by plaque assay or automated fluorometry (16).

Transfection, Selection of Transfected Cells, and Generation of Cell Clones—Transient Lipofectamine Plus transfection (Invitrogen) of 293 and HeLa cells was performed at a cell confluence of 75%. HFFs were generated with vectors pcDNA3.1, pcDNA5/FRT (Invitrogen); pMACS Kk.II. PCR was performed with vectors pGBT9, respectively, and subsequent insertion into vectors pGBT9 and pACT. Deletion mutants of pUL97 and p32 were generated by the protocol of the manufacturer (Invitrogen).

Construction of Expression Plasmids—Expression plasmids were generated with vectors pcDNA3.1, pcDNA5/FRT (Invitrogen); pMACS Ks.II (Milenyi Biotech); pGEX-6p-1 (Amersham Biosciences); pACT, pAS1, and pGBT9 (Clontech). The integrity of virus stocks.

Construction of Recombinant Virus—UL97-deleted HCMV, expressing green fluorescent protein (GFP) as a reporter of virus replication, was generated by the use of the BACmid technology as described previously (18–20). Recombination plasmid pST-GFPneo was constructed by insertion of a 7.1-kb NheI/ClaI fragment containing GFPneo from pHB5 (19), the fragment was inserted into the US9/US10 region of the baculovirus genome.

Yeast Two-hybrid Screening—Protein interactions were analyzed using GAL4 fusion proteins in the yeast two-hybrid system (21). Selection for the presence of bait and interactor plasmids was achieved by cultivation on media restricting growth for combined tryptophan/leucine prototrophy. Primary transformants were selected for growth on histidine-deficient plates containing 30 mm 3-amino triazole. His-selected colonies were analyzed for β-galactosidase activity by filter lift tests. Yeast DNA was isolated from positive clones, and interactor plasmids were rescued by transformation of E. coli strain KC8. Expression was confirmed by Western blot analysis using monoclonal antibodies mAb-GAL4BD and mAb-GAL4AD (Clontech). Plasmids were retrotransformed into yeast to confirm interaction before sequences of the cDNA inserts were determined by automated sequence analysis (Applied Biosystems).

Coimmunoprecipitation (CoIP) Analysis—Infected HFFs or transfected 293 cell lines (~5 × 10⁶ cells) were lysed in 1 ml of CoIP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin) and used for CoIP with mAbs (2) for 2 h at 4 °C under rotation. For CoIP of LBR from HCMV-infected HFFs (see Fig. 4B), the nuclear lamina fraction was enriched by exclusion of nuclei in hypotonic buffer A (10 mM KCl, 0.1% Nonidet P-40, 10 mM HEPES pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) followed by

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centrifugation through a 25% sucrose cushion and lysis in CoIP buffer. Protein A-Sepharose beads were added to the CoIP reactions (2.5 mg, 2 h at 4 °C, Amersham Biosciences) before complexes were pelleted, washed, and used for separation in 12.5% SDS-PAGE followed by Western blot analysis (mAb-FLAG M2, Sigma), anti-HA.11 (Babco) mAb-UL97, anti-LBR, and anti-p32 antibodies; ECL staining, New England Biolabs).

In Vitro Kinase Assay—The kinase activity of pUL97 was determined in vitro after immunoprecipitation from transfected 293 cells as described (17). Putative phosphorylation substrates were either added exogenously as purified proteins (GST-p32; histone 2B, Roche Applied Science) or simultaneously immunoprecipitated from cell lysates using specific antibodies (polyclonal rabbit anti-p32, provided by W. C. Russell, U. K.; guinea pig polyclonal anti-LBR, provided by H. Herrmann, DKFZ Heidelberg; mouse mAb-lamin A/C (636) and polyclonal goat anti-lamin B (M-20), Santa Cruz Biotechnology, Santa Cruz, CA).

Indirect Immunofluorescence Double Staining—HeLa cells or HFFs were grown on coverslips for transfection or HCMV infection, respectively. 2 days post-transfection or at the indicated time points postinfection, cells were fixed with ice-cold methanol for 10 min. Primary antibodies were incubated for 90 min at 37 °C. Secondary antibodies were used for double staining in green (anti-rabbit-FITC; Dianova) and red fluorescence (anti-mouse-Cy3; Dianova), by incubation for 45 min at 37 °C (nuclear counterstaining with DAPI Vectashield mounting medium; Vector Laboratories). Data for immunofluorescence were collected using an Axiovert-135 microscope at magnifications of 400 and 630 × (Zeiss). Images were recorded with a Cooled Spot Color Digital Camera (Diagnostic Instruments). Three-dimensional deconvolution microscopy was performed with an Axiovert-135 microscope equipped with Z-motor and motorized filter wheel (Visirtron Systems, Puchheim, Germany). Metavue software was used to generate Z-series, and processing of the images was achieved with AutoDeblur (AutoQant Imaging: Watervliet, NY).

Analysis of HCMV Production and Release—U373 cell clones or HFFs were used for infection with HCMV AD169 or AD169-GFP and analyzed by indirect immunofluorescence staining (detection of major capsid protein-producing cells; mAb-MCP) or automated GFP fluorometry (performed with lysates of infected cells; 16). Supernatants from HCMV-infected cells were taken at consecutive times postinfection and purified from cell debris. Virus particles were pelleted by centrifugation for 2 h at 25,000 × g at 4 °C and analyzed by 12.5% SDS-PAGE and Western blot staining (mAb-pp65). For the determination of infectious virus, supernatant samples were used for plaque assay titration on HFFs using a 0.3% agar overlay. Staining of plaques was performed with 1% crystal violet at 8–12 dpi.

RESULTS

pUL97 Induces Alterations in the Subnuclear Localization Pattern of Lamin Components—pUL97 is required for an efficient nuclear export of viral particles (15). Because the nuclear lamina is a natural barrier for nucleocytoplasmic transition independent of the nuclear pore complex, we investigated the morphology of the nuclear lamina in pUL97-expressing cells. First we asked for possible alterations in the typical staining pattern and subnuclear localization of the lamina components. For this, we used an expression construct for an N-terminal fragment including the first transmembrane domain of the LBR (amino acids 1–238) fused to GFP (23). This expression construct had been used previously for studies on HSV-infected cells showing that the LBR-GFP fusion protein is incorporated into the nuclear lamina and is redistributed in the context of viral infection (1). In our experiments, LBR-GFP showed a distinct nuclear rim staining (Fig. 1A, b). Upon pUL97 coexpression, however, the membrane-associated appearance of LBR-GFP largely disappeared and was converted to a granular intranuclear structure (Fig. 1A, e). This process was dependent on the kinase activity of pUL97 because a catalytically inactive point mutant of pUL97 did not induce a comparable effect (Fig. 1A, h). Second, immunofluorescence staining of endogenous lamin A/C confirmed the morphological alteration of lamina components. In the presence of active pUL97 kinase (but not the inactive mutant) lamin A/C disappeared from the nuclear rim and was redistributed in a fashion similar to that observed for LBR-GFP (Fig. 1B, b). An examination of the fine structure of the lamina was performed by three-dimensional deconvolution microscopy (Fig. 1C, a–f). As investigated for serial levels of the cell body, intact lamin A/C rim staining was almost nondetectable in pUL97-expressing cells (Fig. 1C, a, left) but constantly detectable in pUL97-negative cells (Fig. 1C, a, right). No comparable difference was observed for a marker protein of the outer nuclear membrane and endoplasmic reticulum (calreticulin; Fig. 1C, d). It should be noted that in transiently UL97-transfected HeLa cells, alterations in the chromatin structure were observed by microscopic analysis in several cases (DAPI staining). This effect might either be associated with the rearrangement of the nuclear lamina or, alternatively, with an induction of apoptosis. Investigation of these cells using a standard assay for a typical early marker of apoptosis (annexin-V-fluos staining kit; Roche Applied Science) and a second assay detecting the activity of cellular proteases associated with apoptosis (PARP assay; Oncogene) did not provide evidence for the latter hypothesis (data not shown). In parallel, Western blot analysis revealed that no fast-migrating degradation forms of LBR and lamins appeared after UL97 transfection or HCMV infection, respectively (data not shown). This supports the conclusion that the observed changes in the subnuclear localization of lamina components is the result of depolymerization rather than proteolytic fragmentation. Interestingly, a link between pUL97 and alterations of the nuclear lamina could also be demonstrated with HCMV (AD169)-infected cells, whereas this effect was absent from cells infected with UL97-deleted HCMV (BAC213; Fig. 1D). Lack of production of pUL97 by BAC213 was controlled by immunofluorescence staining (Fig. 1D, g). Both viruses produced MCP, a true late marker of the replication cycle (Fig. 1D, b and h). Viral pUL84 was used as an early marker of replication for double stainings with lamin A/C in infected cells. Although AD169 replication was associated with a complete loss of signals for lamin A/C (Fig. 1Dc–f), the normal lamin A/C morphology was maintained in BAC213-infected, pUL84-positive cells (Fig. 1D, i–m). This indicates that lamin A/C relocalization may not occur in the absence of pUL97. Furthermore, in HCMV (AD169-GFP)-infected cells, a drastic reduction in the detectable amount of lamins A/C could be identified on Western blots (Fig. 1E, middle panels). This reduction was clearly dependent on virus replication as illustrated by increasing m.o.i. values (which were verified by GFP microscopy and the detection of pUL97 on Western blots; Fig. 1E, top panels). The loss of immunoreactivity of lamin A (which is not caused by proteolysis) is a late event during the course of HCMV replication as described previously by Radask et al. (24). In our analysis, a general alteration in the amount of cellular protein in virus-infected cells could be excluded by the control staining of constant levels of β-actin (Fig. 1E, bottom panels). Hyperphosphorylation of lamins possibly leading to loss of recognition by the monoclonal antibody provides an obvious explanation. Most important seems the finding that this effect is not detectable in cells infected with UL97-deleted HCMV (BAC213). Thus, immunoreactivity of lamins A/C is not altered in BAC213-infected cells. This finding further underlines the importance of pUL97 for the lamin relocalization phenotype of HCMV replication.
pUL97 Interacts with the Lamina-associated Cellular Protein p32—Based on the observation shown in Fig. 1, we tried to identify cellular pUL97-interacting proteins, in particular components of the nuclear lamina. These interactors might either be phosphorylated targets (typically low affinity interaction) and/or might undergo protein-protein binding with pUL97 to influence its activity (possibly high affinity interaction). First, we performed a biochemical investigation of lamin proteins using lamin-specific antibodies to determine possible pUL97-specific effects. However, neither in vitro kinase assays with lamin immunoprecipitated from lysates of pUL97-expressing cells nor attempts to coimmunoprecipitate putative targets together with pUL97 (using mAb-FLAG) were successful. Thus, with these approaches we were unable to detect an in vitro phosphorylation of lamins or a direct interaction between pUL97 and lamins A/C. Therefore, a yeast two-hybrid screening was performed with a human cDNA library (22). Among 1.2/100,000 yeast transformants, 461 auxotrophy-selected colonies were isolated. Binding activity of these initial clones was analyzed by filter lift β-galactosidase assays and confirmed by retransforming yeast with the plasmids. Finally, 12 clones were confirmed to be positive for β-galactosidase activity in filter lift and o-nitrophenyl...
galactosyltransferase assay. Interestingly, after sequence analysis no classical component of the nuclear lamina was identified as a putative pUL97 interaction partner. However, four clones were identified as coding sequences of human p32 (amino acids 1–282, 5–282, 15–282, and 50–282; Fig. 2A). p32 is known as a multifunctional protein in various cell types (25; also referred to as HABP1, TAP, and pC1qR) and was described recently as a lamin-associated protein undergoing direct protein interaction with LBR (26–28). In specificity controls, these p32 clones did not produce interaction signals with a cellular reference protein (p53) but showed strong interaction not only with wild-type but also mutants of pUL97 (catalytically inactive K355M or ganciclovir phosphorylation-deficient M4608 mutant; data not shown). This indicates that kinase activity is not a prerequisite for p32 interaction. To map regions essential for interaction between p32 and pUL97, N-terminal and C-terminal deletion mutants of p32 were generated (Fig. 2A). N-terminal deletion was tolerated to amino acid 50, whereas all C-terminal deletion mutants were negative. A short C-terminal fragment (214–282) showed an intermediate level of interaction signal. Furthermore, a randomly isolated point mutation in the C-terminal part of clones p32(50–282/L243H)F and p32(1–282/L243H)F completely abrogated interaction. Interestingly, although lysine 243 in p32 was essential for interaction with pUL97, mutation L243H in p32 did not prevent interaction with wild-type p32. Positive signals for interaction of p32 with itself in the yeast system are consistent with the previously described multimerization activity of p32 in mammalian cells (29, 30). The finding in Fig. 2A suggests that p32 multimerization and interaction of p32 with pUL97 require distinguishable interaction sequences and/or structural properties. Thus, the optimal pUL97-binding region is p32 fragment 50–282 (suggesting a strong structural requirement for high affinity binding), and the C-terminal 69 amino acids are essential for interaction. Further analysis with the yeast two-hybrid system was performed using N-terminal and C-terminal deletion mutants of pUL97 to map a putative interaction domain. The boundaries of the region involved in p32 interaction are defined by fragment 181–365 (Fig. 2B).

pUL97 and p32 Interact through a Common Interaction Domain—pUL97 immunoprecipitates with p32 from infected or transfected cells, and the Two Proteins Colocalize at the Nuclear Lamina—In human fibroblasts infected with low or high m.o.i. of HCMV, pUL97 was detectable by Western blot analysis (Fig. 3A, lanes 5 and 6). After CoIP with anti-p32 antiserum, a specific interaction signal for pUL97 was obtained (Fig. 3A, lanes 2 and 3). This reaction was negative in a control experiment using a virus mutant lacking pUL97 expression (11; data not shown). Thus, pUL97 interacts with endogenous p32 in infected fibroblasts. In transfection experiments, pUL97 was sufficient to coimmunoprecipitate with endogenous p32 in the absence of other viral proteins (Fig. 3B, lane 5). The catalytically inactive mutant pUL97(K355M)F was also positive for interaction (Fig. 3B, lane 6). No interaction was noted for the viral early-late tegument protein pUL26F (lane 7). The known cellular ligand of p32, splicing factor SF2 (25, 31), served as a positive control (Fig. 3B, lane 8). Importantly, pUL97 fragment 181–365 was also coimmunoprecipitated with anti-p32 antiserum (lane 10), confirming the mapping of this interaction region shown in Fig. 2B. It should be mentioned that interaction region 185–365 overlaps with the ATP binding site of the pUL97 kinase domain (essential lysine 355). This may suggest a modulatory effect of this interaction on pUL97 kinase activity (see also Fig. 5C). The interaction of pUL97 with p32 was further analyzed by immunofluorescence double staining of pUL97-transfected cells. Endogenous p32 partly localized in the nucleus and/or cytoplasm, whereby the latter finding was consistent with the known mitochondrial translocation of p32. Notably in those cells, in which p32 was distinctly localized in the nucleus, a punctate staining pattern lining the nuclear envelope was observed. Interestingly, in these locations p32 clearly colocalized with pUL97F (Fig. 3C, a–d). This characteristic rim staining and colocalization with p32 were also obtained with deletion mutants pUL97(1–459)F and pUL97(1–365)F (Fig. 3C, e and f), whereas a mutant lacking the interaction domain failed to colocalize (pUL97(366–707)F, Fig. 3C, g). A control staining with a lamin B-specific antibody (Fig. 3C, h) visualized colocalization with p32, thus suggesting the nuclear lamina as the main site of p32–pUL97 colocalization.

p32 Interacts with the Lamin B Receptor in Human Cells and Recruits pUL97 to Form a Trimeric Protein Complex—The inner nuclear membrane contains specific resident proteins including LBR, which serves as a contact point for components of the nuclear lamina and lamina-associated proteins. Interestingly, a direct interaction between LBR and p32 had been demonstrated for avian cells (27, 28), and interaction between
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The postulated role of p32 as a factor mediating pUL97 protein interactions is consistent with these data. pUL97 Phosphorylates p32 and Components of the Nuclear Lamina—p32 is subject to phosphorylation by cellular kinases in vivo (32). To investigate whether p32 is also a phosphorylated substrate of viral pUL97, in vitro kinase assays were performed. To this end, p32 was produced in bacteria, purified as a GST fusion protein, and exogenously added to a kinase reaction with pUL97 which was immunoprecipitated from transfected 293 cells. A clear phosphorylation signal was obtained for GST-p32 (Fig. 5A, lane 2), whereas no signals were detected for GST alone (Fig. 5A, lane 3) and a nonrelated protein (CREB; Fig. 5A, lane 4). No phosphorylation of GST-p32 was detected with the catalytically inactive K355M mutant of pUL97, excluding the possibility that an associated cellular protein kinase was coimmunoprecipitated and responsible for the effect (Fig. 5A, lane 5). To map the substrate recognition domain in pUL97 responsible for p32 phosphorylation (which was expected in the region 181–365), several deletion mutants were analyzed (Fig. 5B). In vitro kinase analysis showed that those pUL97 mutants lacking autophosphorylation (C-terminal mutants and N-terminal deletion 366–707; Fig. 5B, lanes 2–6) were inactive in the phosphorylation of p32. Thus, the C-terminal portion of pUL97 (including amino acids 596–707) is essential for its

p32 and recombinant versions of human LBR was shown recently by Mylonis et al. (26). Thus, we asked whether the native human LBR interacts with p32 in HeLa cells and HCMV-permissive HFFs. To this end, lysates of transiently transfected HeLa cells overexpressing p32F (Fig. 4A, top panel) were used for CoIP analysis. When p32F was immunoprecipitated with mAb-FLAG, LBR was detected in the coimmunoprecipitate using a specific antiserum against the LBR (Fig. 4A, bottom panel, lane 2). In addition, pUL97Y11A produced by cotransfection was also coimmunoprecipitated and detectable together with p32F and LBR (Fig. 4A, middle panel, lane 3) suggesting the formation of a trimeric complex. This suggestion was confirmed by analyzing coimmunoprecipitates derived from HCMV-infected HFFs. As demonstrated for serial m.o.i. values (Fig. 4B, top panel), pUL97 was specifically coprecipitated with anti-LBR antiserum (Fig. 4B, middle panel, lanes 2–4), whereas preimmune serum was negative (Fig. 4B, middle panel, lane 5). Furthermore, CoIP of p32 was also detectable, and signals increased with higher m.o.i. values, indicating that the protein complex is stabilized in the presence of pUL97 (Fig. 4B, bottom panel). Interestingly, in a yeast two-hybrid experiment performed in parallel, no direct interaction between pUL97 and LBR (N-terminal fragment 1–208) could be observed, whereas the interaction between p32 and LBR could be confirmed (data not shown). These experiments strongly suggest the formation of a trimeric complex pUL97-p32-LBR in HCMV-infected cells.
C (Fig. 5)

FIGURE 4. Interaction among p32, pUL97, and LBR. A, 293 cells were transfected with p32 alone (top panel, lane 2; expression control Western blot, mAb-FLAG) or in combination with pUL97 (lane 3). Immunoprecipitation of p32 was performed with mAb-FLAG, and coimmunoprecipitates were analyzed for the presence of pUL97 (middle panel, rabbit anti-HA) and p38 (bottom panel, guinea pig anti-LBR). 8, HFFs were infected with HCMV AD169-GFP at m.o.i. values of 0.1, 0.25, 0.5, and 1 or remained unaffected and were harvested 4 days postinfection. Infection controls were assayed on Western blots (mAb-UL97, top panel). Lysates were subjected to CoIP with guinea pig anti-LBR antiserum (lanes 1–4) or preimmune serum (lane 5). Coimmunoprecipitates were detected on Western blots (middle panel, mAb-UL97; bottom panel, rabbit anti-p32).

Overall catalytic activity, and the postulated p32 recognition domain (181–365) is linked to an essential region (ATP binding site) of the pUL97 kinase domain. Next, we analyzed the phosphorylation of purified histone 2B (H2B) in the presence and absence of GST-p32. H2B is an in vitro standard substrate of pUL97 and many other serine/threonine-specific protein kinases. As shown in Fig. 5C, the efficiency of H2B phosphorylation was significantly lower in the presence of GST-p32 compared with the control with GST alone. In contrast, the phosphorylation of GST-p32 and autophosphorylation of pUL97 were not considerably influenced by the addition of H2B but remained at a constant level. Thus, p32 might be a preferential substrate compared with H2B. Alternatively, it seems suggestive that the substrate specificity of pUL97 is changed after binding of p32. This might favor the phosphorylation of p32 and possibly other substrates of pUL97. Therefore, we searched for further phosphorylated cellular targets of pUL97, especially those associated with the nuclear lamina. Depolymerization of the nuclear lamina is a typical feature of herpesvirus infections (1, 5, 24) as well as cellular mitosis (3, 4, 27). In both cases, phosphorylation of lamins is an important trigger of the remodeling process. To investigate this point for HCMV-infected cells, pUL97-specific phosphorylation of lamins was analyzed. As an important result, pUL97 catalyzes the phosphorylation of lamins. Reaction was positive for lamin isotypes A, B, and C (Fig. 5D, lanes 3 and 6). Lamin phosphorylation was directly mediated by pUL97 because the use of the catalytically inactive mutant pUL97(K355M) as a specificity control did not result in phosphorylation signals (data not shown). Moreover, phosphorylation of p32 by pUL97 could also be demonstrated (Fig. 5D, lane 8). In contrast, phosphorylation of LBR was not detectable (Fig. 5D, lane 7). The latter point was further addressed by analyzing coimmunoprecipitates obtained with various p32- or pUL97-specific antibodies from p32-/pUL97-overexpressing cells. In no case could a LBR-specific phosphoband be identified in in vitro kinase assays, suggesting that LBR is not a physiological substrate of pUL97. Thus, these findings demonstrate that a herpesviral protein kinase can phosphorylate individual components of the nuclear lamina.

Overexpression of p32 Increases the Replication Efficiency and the Release of HCMV—The functional relevance of p32 expression during HCMV replication should be clarified. Because the level of endogenously expressed p32 varies among cell types and is comparatively low in HCMV-permissive fibroblasts, we performed two different approaches to increase p32 levels in HFF and U373 cells. First, we overexpressed p32 in stably transfected cells. A previously generated clone of U373 cells (U373FRT), harboring the Flp recombinase site at a transcriptionally active position of the cellular genome was used to insert an expression module for p32. Insertion of the expression module at the designated integration site was proven by PCR, and overexpression of p32 was demonstrated by Western blot analysis (Fig. 6A). p32 was expressed at levels 3–5-fold higher than endogenous p32. It is noteworthy to mention that U373 cells are generally limited in the production of HCMV progeny and provide a semipermissive phenotype for HCMV replication. In infection experiments with HCMV AD169-GFP, which expresses GFP under control of the HCMV IE-promoter, virus uptake was monitored by fluorescence microscopy detecting initial GFP expression within the early phase of infection (1–2 dpi). At this stage, no differences were noted between individual cell clones (data not shown). In contrast, at later time points (second round of virus replication, 5 dpi), infected cell clones showed markedly different quantities of GFP signals as assayed by automated fluorometry (Fig. 6B). Cell clones expressing p32 (clone C) and N-terminally truncated p32(50–282) both supported a highly increased efficiency of virus production (~30-fold at GFP-TCD50 multiplicity 1) compared with the parental cell clone U373 (clone E2). It should be mentioned that the N-terminally truncated p32(50–282) resembles the naturally occur-

5 M. Leis, unpublished data.
ring cleavage product of p32 lacking an N-terminal mitochondrial targeting signal (33). Interestingly, the cell clone expressing point mutant L243H (negative for interaction with pUL97) showed an intermediate level of virus replication (Fig. 6B, right panel). Replication in U373-p32(L243H)F cells was significantly less effective than in U373-p32F (C) but surprisingly more effective than in U373 (E2) cells. This finding might be explained by the capacity of p32 to form trimers and higher multimers (33). As demonstrated by the yeast two-hybrid system, mutant p32(L243H)F retained the ability to interact with p32 wild-type. This suggests that ectopically expressed p32(L243H)F seems to contribute to an increased amount of overall p32 multimer formation, which might promote pUL97 interaction to a higher extent than that mediated through endogenous levels of p32 in control cells. Thus, interaction between p32 and pUL97 positively influences the HCMV replication efficiency. Second, we expressed p32F in a vector system, which enables the enrichment of positively transfected primary HFFs using magnetic beads (Fig. 6C). Selected cell populations were used for infection with HCMV AD169-GFP and for quantification of virus replication at 7 dpi. HFFs overexpressing p32F or p32(50–282)F produced significantly higher amounts of HCMV than vector-transfected control cells. The results are consistent with those obtained with stably p32F-overexpressing U373 clones, thus confirming the stimulatory effect of p32 on HCMV replication. Next, we addressed the question of whether the level of p32 quantitatively correlates with release of progeny virus. U373-p32F (clones A and C) and parental U373 cells (clones E2 and E4) were used for infection with HCMV (Fig. 6D). Virus released to the supernatants (indicated by Western blot staining of tegument protein pp65) was detected at 10–12 dpi only in clones A and C, but not in clones E2 and E4. Furthermore, the quantity of infectious virus in these supernatants was determined by plaque titration. An increase in the release of infectious virus was noted for clones A and C, at time points later than 6 dpi (3-fold increase at day 8) but not for clones E2 and E4 (Fig. 6E; an initial peak at 2 dpi is explained by the detachment of inoculum virus). In contrast to virus release, no significant difference in the production of viral capsids (indicated by fluorescence immunostaining of cells producing MCP) was found for clones A, C, E2, and E4 (Fig. 6F). Thus, p32 is a cellular factor determining the efficiency of HCMV particle release. Taken together these results are
consistent with the idea that p32 recruits pUL97 to the LBR for the phosphorylation of lamins. This might largely contribute to the dissolution of the nuclear lamina as a crucial step determining the efficiency of viral nuclear capsid export.

**DISCUSSION**

The protein kinase pUL97 of HCMV is required at the stage of nuclear egress of viral particles (15). We identified an altered pattern of lamina morphology upon pUL97 overexpression and searched for cellular interaction partners to gain insight into the responsible mechanisms. Here we report that (i) pUL97 interacts directly with cellular p32; (ii) the complex between pUL97 and p32 accumulates at the nuclear lamina; (iii) p32 mediates the recruitment of pUL97 to the lamina by interaction with the LBR; (iv) pUL97 phosphorylates p32 as well as nuclear lamins; (v) the phosphorylating activity of pUL97 is required for alterations of the lamina morphology; and (vi) overexpression of p32 results in an increased efficiency of HCMV replication and release.

p32 was originally described as an interactor of splicing factor SF2/
we generated a deletion mutant of pUL97, which fails to interact with cellular protein kinase activities may additionally be involved. Recently, because pUL97 is not absolutely essential for viral replication, we found predominantly in a cytoplasmic or mitochondrial localization. In addition, several viral proteins were identified as interactors of p32, among them important determinants of viral replication efficiency (e.g. HSV-1 IP27 (36); Epstein-Barr virus nuclear antigen-1 (37); rubella virus capsid protein (38); HIV-1 Tat and Rev (39, 40)). The variety of p32 interactions with a series of proteins encoded by human pathogenic viruses illustrates its multifunctional mode of action. Of major importance seems the finding that p32 is a determinant of HIV-1 replication, particularly contributing to restriction of the viral tropism to human cells. A post-transcriptional block described for HIV-1 in murine cells could be partially relieved by ectopic overexpression of human p32 (41).

With respect to the proviral effect of p32 shown in the present study, it is important that p32, which can be found in variable intracellular localizations, is in part associated with the LBR complex (26, 28, 42). As shown in detail for avian cells, LBR (p58) forms functional complexes with the avian homolog of p32 (p34), nuclear lamin B, the integral membrane protein p18, the RS kinase, and possibly other components. Interestingly, phosphorylation of LBR by the RS kinase abolishes the binding of p32 (p34), suggesting that protein kinase activity regulates the interactions among components of the LBR complex (27). During the late phase of replication of herpesviruses, viral capsids undergo nucleocytoplasmic egress (43) and bud through the inner and outer membranes of the nuclear envelope, which is structurally tightened by the rigid nuclear lamina meshwork. Structural destabilization of the nuclear lamina is generally mediated through phosphorylation of nuclear lamins (2). In HCMV-infected cells, alterations of the nuclear lamina were described (24, 44); however, the identity of protein kinases involved in lamin phosphorylation has remained speculative so far. Our data provide evidence for the recruitment of a virus-encoded protein kinase to the nuclear lamina. Importantly, pUL97 possesses lamin phosphorylating activity and is detectable in the perinuclear space of infected cells (for additional description, see Ref. 10). Furthermore, overexpression of pUL97 in the absence of other viral proteins leads to an alteration of the subnuclear localization of LBR and lamins A/C. Thus, we conclude that pUL97 coregulates the dissolution of the nuclear lamina in infected cells. However, because pUL97 is not absolutely essential for viral replication, cellular protein kinase activities may additionally be involved. Recently we generated a deletion mutant of pUL97, which fails to interact with p32 but retains full protein kinase activity (pUL97(231–707))8. This mutant was analyzed in transfected HeLa cells for its lamin relocalization phenotype. Although we did not find a complete disruption of this phenotype, a clear quantitative reduction in altering the nuclear lamina was observed compared with pUL97 wild-type.9 This finding provides an additional indication for the importance of the kinase activity of pUL97 in lamin relocalization and particularly for the enhancing effect of interactor p32.

Generally in the course of herpesvirus replication, the nucleocytoplasmic capsid export through the nuclear envelope represents a rate-limiting step. In HSV-infected cells, a specific redistribution of LBR to the endoplasmic reticulum accompanied by the dissolution of the rim structure of the nuclear envelope was demonstrated (1). Regulatory proteins directly involved in capsid egress were described in detail for HSV-1 as well as pseudorabies virus, and their combined action (i.e. UL34, UL31, and US3) has been illustrated by several studies (6, 7, 45). However, the mechanism of lamina destabilization still remains to be clarified. In case of murine cytomegalovirus infection, the effect of cellular protein kinase C on the nuclear lamina was reported (5). Here, the viral proteins M50/p35 and M53/p38 (which are homologs of HSV-1 UL34 and UL31) play an important role by recruiting protein kinase C for the phosphorylation of lamins and by forming a docking site for viral capsids. Our study for the first time describes the recruitment of a herpesviral protein kinase to the nuclear lamina for lamin phosphorylation and redistribution, which is functionally connected with viral nuclear capsid export. Further investigations are necessary to understand the postulated combined action of viral and cellular protein kinases at the nuclear lamina. Particularly, the description of protein complexes combining viral and cellular components, such as pUL97-p32-LBR, may provide novel molecular targets for antiviral therapy.

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Cellular p32 Recruits Cytomegalovirus Kinase pUL97 to Redistribute the Nuclear Lamina

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